

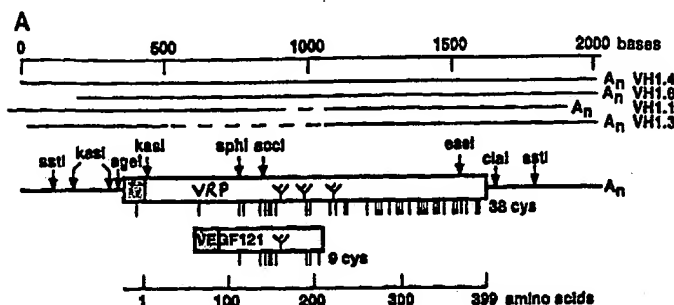
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/19, C07K 14/52, 19/00, 16/24, A61K 38/19, G01N 33/50		A1	(11) International Publication Number: WO 97/09427
			(43) International Publication Date: 13 March 1997 (13.03.97)
(21) International Application Number: PCT/US96/14075		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 30 August 1996 (30.08.96)		<p>Published</p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
(30) Priority Data: 60/003,491 8 September 1995 (08.09.95) US			
(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).			
(72) Inventors: LEE, James; 705 Shelter Creek Lane, San Bruno, CA 94066 (US). WOOD, William; 1400 Tarrytown Street, San Mateo, CA 94402 (US).			
(74) Agents: LEE, Wendy, M. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).			

(54) Title: VEGF-RELATED PROTEIN

(57) Abstract

A human VEGF-related protein (VRP) has been identified and isolated that binds to, and stimulates the phosphorylation of, the receptor tyrosine kinase Flt4. The VRP is postulated to be a third member of the VEGF protein family. Also provided are antibodies that bind to VRP and neutralize a biological activity of VRP, compositions containing the VRP or antibody, methods of use, chimeric polypeptides, and a signal polypeptide for VRP.



B

VRP -30 MHLGGFFSVACBLLAAALLPPPREAPAAAAAFESOLDLSDAEPDAGEATA
 VRP 31 YASKDLLEQLREVBVDELWTVLYPEYKKYKCOARKQGNHNRQDNLN
 VEGF121 -20MFLLEWVHWSLALLYLHHA
 PIGF121 -10PVMRFPGFLDLAAGLPLA
 VRP 81 SRTEETIKFKAANYTEILKSIDNEARKTOENPVEVCIVOKFEQVATNT
 VEGF121 -5 KWSAAPMAEDGDDHMHVEVYFNDFYORSTYCNIEITVOLFQETPDEIEY
 PIGF121 4 VPPDQWALSGDNSSVEVYFEOEYWRSSYCALERLVYVSVSEYPSVEVH
 VRP 131 FFKPPEVYVYRCCGCCNREGLOPNNFISYLYLKYTFETVPLSPGKPKPT
 VEGF121 48 IFKPTGVPTMRCQGCCNDEGLQVPTIEEYITFQITMRKPT...HSDQHIG
 PIGF121 84 MEFPKCVSLRGTCCGDENLKCYVETANVYVMDLKLRS...GDRPSTVE
 VRP 181 IFIANHTSDFMSKLDVYRQVMSIIRSLPATLPQGAANKTCPTNYMWN
 VEGF121 94 MEFLDNNKCTCTKKAALQKPKPR
 PIGF121 102 LTFSDHVRCEGRPLREKMKPERDQDAVPRR
 VRP 231 NHICRCLAGEDFMFSSDAAGDDSTQDFNDICGPNKELDEETCCQVCRAQLR
 VRP 281 PASCQPHKELDRNSCGQVCKNKLFPSCQGANREFDENTCCQVCKRTCPRN
 VRP 331 QPLNPQKCAECTESPQKOLLKQKFNHOTSCVRRPCTNRQKACEPGFS
 VRP 381 YSEEVQRCVPBYWKRPGMS

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

VEGF-RELATED PROTEIN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention pertains generally to a receptor protein tyrosine kinase (rPTK) ligand. More particularly, the invention relates to a novel ligand, designated VEGF-related protein (VRP) or VHI, which binds to, and stimulates the phosphorylation of, the Flt4 tyrosine kinase receptor (also known as the Sal-S1 receptor) and the isolation and recombinant production of the same.

Description of Related Art

The formation of new blood vessels either from differentiating endothelial cells during embryonic development (vasculogenesis) or from pre-existing vessels during adult life (angiogenesis) is an essential feature of organ development, reproduction, and wound healing in higher organisms. Folkman and Shing, *J. Biol. Chem.*, 267: 10931-10934 (1992); Reynolds *et al.*, *FASEB J.*, 6: 886-892 (1992); Risau *et al.*, *Development*, 102: 471-478 (1988). Angiogenesis is also necessary for certain pathological processes including tumorigenesis (Folkman, *Nature Medicine*, 1: 27-31 [1995]) and retinopathy. Miller *et al.*, *Am. J. Pathol.*, 145: 574-584 (1994).

While several growth factors can stimulate angiogenesis (Klagsbrun and D'Amore, *Ann. Rev. Physiol.*, 53: 217-239 [1991]; Folkman and Klagsbrun, *Science*, 235: 442-447 [1987]), vascular endothelial growth factor (VEGF) (Ferrara *et al.*, *Endo. Rev.*, 13: 18-32 [1992]) is a potent angiogenic factor that acts via the endothelial cell-specific receptor tyrosine kinases *fms*-like tyrosine kinase (Flt1) (Shibuya *et al.*, *Oncogene*, 5: 519-524 [1990]; deVries *et al.*, *Science*, 255: 989-991 [1992]) and fetal liver kinase (Flk1) (also designated KDR). Quinn *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 7533-7537 (1993); Millauer *et al.*, *Cell*, 72: 835-846 (1993); Matthews *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 9026-9030 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.*, 187: 1579-1586 (1992); Terman *et al.*, *Oncogene*, 6: 1677-1683 (1991); Oelrichs *et al.*, *Oncogene*, 8: 11-18 (1993). These two VEGF receptors and a third orphan receptor, Flt4 (Pajusola *et al.*, *Cancer Res.*, 52: 5738-5743 [1992]; Galland *et al.*, *Oncogene*, 8: 1233-1240 [1993]; Finnerty *et al.*, *Oncogene*, 8: 2293-2298 [1993]) constitute a subfamily of class III receptor tyrosine kinases that contain seven extracellular immunoglobulin-like domains and a split intracellular tyrosine kinase domain. Mustonen and Alitalo, *J. Cell. Biol.*, 129: 895-898 (1995). See also WO 94/10202 published 11 May 1994 and PCT/US93/00586 filed 22 January 1993 (Avraham *et al.*). These three receptors have 31-36% amino acid identity in their extracellular ligand-binding domains.

Mice deficient in Flt1 (Fong *et al.*, *Nature*, 376: 66-70 [1995]) or Flk1 (Shalaby *et al.*, *Nature*, 376: 62-66 [1995]) (generated by gene targeting in embryonic stem cells) have severe defects in vasculogenesis and die in utero at embryonic day 8-9. The phenotype of the receptor-deficient mice differs considerably, however. Mice lacking Flt1 have a disorganized vascular endothelium that extends to the major vessels as well as to the microvasculature, while endothelial cell differentiation appears to be normal. Fong *et al.*, *supra*. Mice lacking Flk1 have a major defect in the development of mature endothelial cells as well as a severe reduction in hematopoietic cell progenitors. Shalaby *et al.*, *supra*. Thus, VEGF may act on endothelial cells at more than one stage of vasculogenesis.

Flt4 is also specifically expressed in endothelial cells; it is first observed in day 8.5 mouse embryos in endothelial cell precursors. Kaipainen *et al.*, *Proc. Natl. Acad. Sci. USA*, 92: 3566-3570 (1995); Kaipainen *et*

al., J. Exp. Med., **178**: 2077-2088 (1993). See also Hatva *et al.*, Am J. Pathol., **146**: 368-378 (1995). As development proceeds, Flt4 expression becomes confined to the venous and lymphatic endothelium and is finally restricted to the lymphatic vessels. Consistent with this finding, adult human tissues show Flt4 expression in lymphatic endothelia while there is a lack of expression in arteries, veins, and capillaries. Kaipainen *et al.*, Proc. Natl. Acad. Sci. USA, *supra*. Clones encoding human and mouse Flt4 have been isolated either by PCR with primers from conserved tyrosine kinase regions (Finnerty *et al.*, *supra*; PCT/US93/00586, *supra*; Aprelikova *et al.*, Cancer Res., **52**: 746-748 [1992]) or by low-stringency hybridization with a Flk2 probe. Galland *et al.*, Genomics, **13**: 475-478 (1992). Alternative splicing of the Flt4 mRNA produces two variants of the protein differing by 65 amino acids at the C-terminus. Pajusola *et al.*, Oncogene, **8**: 2931-2937 (1993). These variants migrate as bands of 170-190 kDa that are partially cleaved proteolytically in the extracellular domain to produce a form of about 125 kDa. Pajusola *et al.*, Oncogene, **8**, *supra*; Pajusola *et al.*, Oncogene, **9**: 3545-3555 (1994). Expression of the longer spliced form of Flt4 as a chimera with the extracellular domain of the CSF-1 receptor shows that the Flt4 intracellular domain can signal a ligand-dependent growth response in rodent fibroblasts. Pajusola *et al.*, Oncogene, **9**, *supra*; Borg *et al.*, Oncogene, **10**: 973-984 (1995). Flt4 has been localized to human chromosome 5q34-q35 (Aprelikova *et al.*, *supra*; Galland *et al.*, Genomics, *supra*); Flt1 and Flk1 are located at 13q12 (Imbert *et al.*, Cytogenet. Cell Genet., **67**: 175-177 [1994]) and 4q12. Sait *et al.*, Cytogenet. Cell Genet., **70**: 145-146 (1995); Spritz *et al.*, Genomics, **22**: 431-436 (1994).

VEGF is a homodimeric, cysteine-rich protein that can occur in at least four forms due to alternative splicing of its mRNA. Ferrara *et al.*, *supra*. While VEGF is a high-affinity ligand for Flt1 and Flk1, it does not bind or activate Flt4. Pajusola *et al.*, Oncogene, **9**, *supra*. The only other closely related member of the VEGF family is placental growth factor (PIGF), which has 47% amino acid identity with VEGF. Maglione *et al.*, Proc. Natl. Acad. Sci. USA, **88**: 9267-9271 (1991). PIGF also occurs in two alternatively spliced forms which differ in the presence or absence of a basic heparin binding domain of 21 amino acids. Maglione *et al.*, Oncogene, **8**: 925-931 (1993); Hauser and Weich, Growth Factors, **2**: 259-268 (1993). PIGF binds to Flt1 but not to Flk1 (Park *et al.*, J. Biol. Chem., **269**: 25646-25654 [1994]); it is believed that its binding to Flt4 has not been determined. PIGF fails to duplicate the capillary endothelial cell mitogenesis or vascular permeability activities of VEGF, suggesting that these activities are mediated by the Flk1 receptor. Park *et al.*, *supra*.

Molecules that modulate the Flk1 receptor or neutralize activation of a VEGF receptor are disclosed in the patent literature. For example, WO 95/21613 published 17 August 1995 discloses compounds that modulate KDR/Flk1 receptor signal transduction so as to regulate and/or modulate vasculogenesis and angiogenesis and disclose using Flk1 to evaluate and screen for drugs and analogs of VEGF involved in Flk1 modulation by either agonist or antagonist activities; WO 95/21865 published 17 August 1995 discloses molecules immunointeractive with animal neuroepithelial kinase (NYK)/Flk1, which molecules can be used to provide agents for treatment, prophylaxis, and diagnosis of an angiogenic-dependent phenotype; and WO 95/21868 published 17 August 1995 discloses monoclonal antibodies that specifically bind to an extracellular domain of a VEGF receptor and neutralize activation of the receptor.

SUMMARY OF THE INVENTION

cDNA clones have now been identified that encode a novel protein, designated VRP, which binds to and stimulates the phosphorylation of the receptor tyrosine kinase Flt4. VRP is related in amino acid sequence to VEGF, but does not interact appreciably with the VEGF receptors, Flt1 and Flk1.

5 In one aspect, the invention provides isolated biologically active human VRP containing at least 265 amino acids. In another aspect, the invention supplies isolated biologically active human VEGF-related protein (VRP) comprising an amino acid sequence comprising at least residues +1 through 29, inclusive, of Figure 1. In further aspect, the invention supplies isolated biologically active human VRP comprising an amino acid sequence shown as residues -20 through 399, inclusive, or residues 1 through 399, inclusive, of Figure 1.

10 The invention also pertains to chimeras comprising the VRP fused to another polypeptide. For example, the invention provides a chimeric polypeptide comprising the VRP fused to a tag polypeptide sequence. An example of such a chimera is epitope-tagged VRP.

In another aspect, the invention provides a composition comprising biologically active VRP and a pharmaceutically acceptable carrier. In a more specific embodiment, the invention provides a pharmaceutical composition useful for promotion of vascular or lymph endothelial cell growth comprising a therapeutically effective amount of the VRP in a pharmaceutically acceptable carrier. In another aspect, this composition further comprises another cell growth factor such as VEGF and/or PDGF.

15 In a further aspect, the invention provides a method of treating vascular tissue and promoting angiogenesis in a mammal comprising administering to the mammal an effective amount of the composition comprising VRP. In another embodiment, the invention provides a method for treating trauma affecting the vascular endothelium comprising administering to a mammal suffering from said trauma an effective amount of the composition containing the VRP. The trauma is, for example, diabetic ulcers or a wound of the blood vessels or heart. In another embodiment, the invention provides a method for treating a dysfunctional state characterized by lack of activation or lack of inhibition of a receptor for VRP in a mammal comprising administering to the mammal an effective amount of the composition containing the VRP.

20 The invention also provides a method which involves contacting the Flt4 receptor with the VRP to cause phosphorylation of the kinase domain thereof. For example, the invention provides a method for stimulating the phosphorylation of a tyrosine kinase domain of a Flt4 receptor comprising contacting an extracellular domain of the Flt4 receptor with the VRP.

25 The invention also provides a monoclonal antibody which binds to the VRP and preferably also neutralizes a biological activity of the protein, one biological activity being characterized as promoting neovascularization or vascular permeability or vascular endothelial cell growth in a mammal. Alternatively or conjunctively, the invention provides a monoclonal antibody which binds to the N-terminal portion from residues -20 through 137, inclusive, or from residues +1 through 137, inclusive, of the amino acid sequence shown in Figure 1. The antibody can be used, for example, to detect the presence of the VRP in a biological sample suspected of having the protein, or to treat patients. The invention contemplates a pharmaceutical composition comprising such antibody and a pharmaceutically acceptable carrier, as well as a method of treating diseases or disorders characterized by undesirable excessive neovascularization or vascular permeability in a mammal comprising administering to said mammal an effective amount of one of the antibodies described above. Further

included by the invention is a method for treating a dysfunctional state characterized by excessive activation or inhibition of a receptor for VRP in a mammal comprising administering to the mammal an effective amount of one of the antibodies described above.

5 In addition, the invention contemplates a peptide consisting of an amino acid sequence shown as residues -20 through -1, inclusive, of Figure 1.

In a further embodiment, the invention provides an isolated nucleic acid molecule encoding VRP or a VRP chimera. In one aspect, the nucleic acid molecule is RNA or DNA that encodes a biologically active VRP or is complementary to nucleic acid sequence encoding such VRP, and remains stably bound to it under stringent conditions. The nucleic acid molecule optionally includes the regions of the nucleic acid sequences of Figure 1 which encode signal sequences. In one embodiment, the nucleic acid sequence is selected from:

10 (a) the coding region of the nucleic acid sequence of Figure 1 that codes for the preprotein from residue -20 to residue 399 or that codes for the mature protein from residue 1 to residue 399 (i.e., nucleotides 372 through 1628, inclusive, or nucleotides 432 through 1628, inclusive, of the nucleic acid sequence shown in Figure 1 as SEQ ID NO: 1); or

15 (b) a sequence corresponding to the sequence of (a) within the scope of degeneracy of the genetic code.

In another aspect, the nucleic acid molecule can be provided in a replicable vector comprising the nucleic acid molecule operably linked to control sequences recognized by a host cell transfected or transformed with the vector. The invention further provides a host cell comprising the vector or the nucleic acid molecule. A method of producing VRP is also provided which comprises culturing a host cell comprising the nucleic acid molecule and recovering the protein from the host cell culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D depict the nucleotide coding sequence (SEQ ID NO: 1), nucleotide complementary sequence (SEQ ID NO: 2), and deduced amino acid sequence (SEQ ID NO: 3) of the human VRP described herein.

25 Figure 2 depicts binding of Flt4/IgG and of Rse/IgG (an unrelated receptor fusion protein) to the human glioma cell line G61, which binding was evaluated by FACS analysis.

Figures 3A and 3B respectively depict a map of cDNA clones encoding human VRP and an alignment of the protein sequence VRP (SEQ ID NO: 3) with that of VEGF₁₂₁ (SEQ ID NO: 4) and PlGF₁₃₁ (SEQ ID NO: 5). Figure 3A shows the extent of four VRP cDNA clones; dashed lines indicate the missing portions of VH1.1 and VH1.3. Arrows indicate restriction enzyme sites; the shaded box indicates the putative secretion signal sequence; the open box indicates the mature protein; Y-type designations within the open box indicate the potential N-linked glycosylation sites; and vertical lines indicate the cysteine residues. A diagram of VEGF₁₂₁ is shown for comparison. The hydropathy plot (Kyle and Doolittle, *J. Mol. Biol.* 157: 105-132 [1982]) is for VRP. In Figure 3B, overlining indicates the region encoded by an expressed sequence tag (EST) (sequence of a portion of a cDNA clone) from GenBank designated HSC1WF111.

35 Figure 4 depicts a map of the cDNA clone for full-length human VRP herein versus eleven known ESTs. The eleven EST partial amino acid sequence fragments are H07991 and H07899 (5' and 3' ends of the same cloned fragment, respectively), H05134 and H05177 (3' and 5' ends of the same cloned fragment, respectively), HSC1WF112 and HSC1WF111 (3' and 5' ends of the same cloned fragment, respectively), T81481

and T81690 (3' and 5' ends of the same cloned fragment, respectively), R77495 (a 3' end of a cloned fragment), and T84377 and T89295 (5' and 3' ends of the same cloned fragment, respectively).

Figure 5 depicts binding of ¹²⁵I-Flt4/IgG to purified VRP. The binding was performed in the absence (-) or presence (+) of 100 nM receptor IgG fusion protein (Fig. 5A) or with increasing concentrations of Flt4/IgG (Fig. 5B).

Figure 6 shows a graph of the cell count of human lung microvascular endothelial cells as a function of the concentration of VEGF or VRP in the cell culture medium to assess and compare mitogenic activity.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

10 In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

"Human VRP" is defined herein to be a polypeptide sequence containing at least residues -20 to 399, inclusive, or residues +1 to 399, inclusive, of the amino acid sequence shown in Figure 1, including residues -5 to 399, inclusive, and residues -4 to 399, inclusive, of the amino acid sequence shown in Figure 1, as well as
15 biologically active deletional, insertional, or substitutional variants of the above sequences having at least 265 amino acids and/or having at least residues +1 through 29, inclusive, of Figure 1. In a preferred embodiment, the protein sequence has at least residues +1 through 137, inclusive, of Figure 1, more preferably at least residues -20 through 29, inclusive, of Figure 1, and most preferably at least residues -20 through 137, inclusive, of Figure 1. In another preferred embodiment, the biologically active variants have a length of 265 to about 450
20 amino acid residues, more preferably about 300-450, even more preferably about 350-450, and most preferably about 399-419 amino acid residues. Another preferred set of variants are variants that are insertional or substitutional variants, or deletional variants where the deletion is in the signal sequence and/or is not in the N-terminal region of the molecule (*i.e.*, residues 1-29, preferably residues 1-137). The definition of VRP excludes all known EST sequences, such as, *e.g.*, H07991, H05134, H05177, HSC1WF112, HSC1WF111, T81481,
25 R77495, H07899, T84377, T81690, and T89295, as well as all forms of VEGF and PlGF.

"Biologically active" for the purposes herein means having the ability to bind to, and stimulate the phosphorylation of, the Flt4 receptor. Generally, the protein will bind to the extracellular domain of the Flt4 receptor and thereby activate or inhibit the intracellular tyrosine kinase domain thereof. Consequently, binding of the protein to the receptor may result in enhancement or inhibition of proliferation and/or differentiation and/or
30 activation of cells having the Flt4 receptor for the VRP *in vivo* or *in vitro*. Binding of the protein to the Flt4 receptor can be determined using conventional techniques, including competitive binding methods, such as RIAs, ELISAs, and other competitive binding assays. Ligand/receptor complexes can be identified using such separation methods as filtration, centrifugation, flow cytometry (see, *e.g.*, Lyman *et al.*, Cell, **75**:1157-1167 [1993]; Urdal *et al.*, J. Biol. Chem., **263**:2870-2877 [1988]; and Gearing *et al.*, EMBO J., **8**:3667-3676 [1989]),
35 and the like. Results from binding studies can be analyzed using any conventional graphical representation of the binding data, such as Scatchard analysis (Scatchard, Ann. NY Acad. Sci., **51**:660-672 [1949]; Goodwin *et al.*, Cell, **73**:447-456 [1993]), and the like. Since the VRP induces phosphorylation of the Flt4 receptor, conventional tyrosine phosphorylation assays, such as the assay described in Example 5 herein, can also be used as an indication of the formation of a Flt4 receptor/VRP complex.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising the entire VRP, or a portion thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VRP. The tag polypeptide preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues).

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein *in situ* within recombinant cells, since at least one component of the VRP natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

"Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

An "isolated" VRP nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the VRP nucleic acid. An isolated VRP nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated VRP nucleic acid molecules therefore are distinguished from the VRP nucleic acid molecule as it exists in natural cells. However, an isolated VRP nucleic acid molecule includes VRP nucleic acid molecules contained in cells that ordinarily express VRP where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The isolated VRP polypeptide, VRP nucleic acid, or VRP antibody may be labeled for diagnostic and probe purposes, using a label as described and defined further below in the discussion on uses of VRP antibodies.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally,

"operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

5 The term "antibody" is used in the broadest sense and specifically covers single anti-VRP monoclonal antibodies (including agonist and antagonist antibodies) and anti-VRP antibody compositions with polyepitopic specificity.

 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical
10 except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

 The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a
15 variable (including hypervariable) domain of an anti-VRP antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage and Lamoyi, in Monoclonal Antibody
20 Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

 Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495
25 (1975), or may be made by recombinant DNA methods. U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty *et al.*, Nature, 348:552-554 (1990), for example.

 "Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding
30 subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by
35 corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are

those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

As used herein, "vascular endothelial cell growth factor," or "VEGF," refers to a mammalian growth factor derived originally from bovine pituitary follicular cells having the amino acid sequence of Fig. 2 of WO 90/13649, and has the human amino acid sequence of Fig. 10 of WO 90/13649. See also U.S. Pat. No. 5,194,596, which discloses bovine VEGF of 120 amino acids and human VEGF of 121 amino acids. The biological activity of native VEGF is capable of promoting selective growth of vascular endothelial cells but not of bovine corneal endothelial cells, lens epithelial cells, adrenal cortex cells, BHK-21 fibroblasts, or keratinocytes.

The expression "trauma affecting the vascular endothelium" refers to trauma, such as injuries, to the blood vessels or heart, including the vascular network of organs, to which an animal or human, preferably a mammal, and most preferably a human, is subjected. Examples of such trauma include wounds, incisions, and ulcers, most preferably diabetic ulcers and wounds or lacerations of the blood vessels or heart. Trauma includes conditions caused by internal events as well as those that are imposed by an extrinsic agent such as a pathogen, which can be improved by promotion of vascular endothelial cell growth. It also refers to the treatment of wounds in which neovascularization or re-endothelialization is required for healing.

"Promotion of vascular or lymph endothelial cell growth" refers to inducing or increasing the growth of vascular or lymph endothelial cells, including human lung microvascular endothelial cells.

"Disorders related to vasculogenesis and angiogenesis" include cancer, diabetes, hemangioma, and Kaposi's sarcoma.

"Diseases or disorders characterized by undesirable excessive neovascularization or vascular permeability" refer to diseases or disorders that include, by way of example, excessive neovascularization, tumors, and especially solid malignant tumors, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation. Examples of diseases or disorders characterized by undesirable excessive vascular permeability include edema associated with brain tumors, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

"Dysfunctional states characterized by excessive activation or inhibition of a receptor for VRP" (such receptor including Flt4) refer to disorders or diseases that would be beneficially treated by providing to a mammal having such a pathological condition an antagonist to VRP, such as a chimera of Flt4 or its extracellular domain (e.g., an IgG fusion with Flt4) or an antibody to VRP.

"Dysfunctional states characterized by lack of activation or lack of inhibition of a receptor for VRP" (such receptor including Flt4) refer to disorders or diseases that would be beneficially treated by providing VRP or a VRP receptor agonist to a mammal with such a pathological condition.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal herein is human.

5 "Effective amount" or "therapeutically effective amount" of the VRP, VRP composition, antibody, or antibody composition is an amount that is effective either to prevent, lessen the worsening of, alleviate, or cure the treated condition. For example, an effective amount of VRP includes that amount which is sufficient to enhance the growth of vascular endothelium *in vivo* or to treat trauma, and an "effective amount" of VRP antibody includes that amount which is sufficient to reduce excess neovascularization and angiogenesis.

10 II. Modes for Carrying Out the Invention

The present invention is based on the discovery of a novel VRP which binds to, and stimulates the phosphorylation of, the Flt4 receptor.

Three approaches were undertaken to identify protein that would bind and stimulate the phosphorylation of the Flt4 receptor. First, the full-length receptor was stably expressed in 293 cells to establish a receptor tyrosine kinase phosphorylation assay of Flt4 activation. This assay was used to screen about 400 cell supernatants and tissue extracts, without positive results.

15 Second, the extracellular domain of the receptor was expressed as a fusion protein with an immunoglobulin Fc domain. By using this fusion protein (Flt4/IgG) to screen cell lines for membrane-bound ligands by FACS analysis, one positive cell line was identified. The human glioma line, G61, gave about a 10-fold shift in peak fluorescence intensity that was specific for Flt4/IgG (Fig. 2). Attempts to expression clone this putative membrane-bound ligand by the transfection of pools of cDNA clones into COS cells followed by screening with labeled Flt4/IgG gave no positives from 640 pools of 1000-5000 clones each. Flt4/IgG was also used to generate polyclonal antisera and monoclonal antibodies that had agonistic activity and that were used to develop the Flt4 tyrosine phosphorylation assay as described in Example 5 below.

20 Third, candidate ligand proteins were tested for their ability to bind to Flt4/IgG or to activate the Flt4 phosphorylation assay. Labeled VEGF failed to bind to Flt4/IgG, although the expected binding of VEGF to Flt1/IgG or Flk1/IgG was routinely detected. The failure of VEGF to bind or stimulate the phosphorylation of Flt4 has been reported by Pajusola *et al.*, *Oncogene*, 2, *supra*. An additional candidate ligand protein was found by use of cloning techniques, details of which are provided in Example 3 below. The human VRP cDNA sequence is depicted in Figure 1A-1D. The predicted molecular weight of the protein is 44.8 kDa.

25 A description follows as to how the biologically active human VRP may be prepared.

30 1. Preparation of VRP

Most of the discussion below pertains to production of VRP by culturing cells transformed with a vector containing VRP nucleic acid and recovering the polypeptide from the cell culture. It is further envisioned that the VRP of this invention may be produced by homologous recombination, as provided for in WO 91/06667, published 16 May 1991.

Briefly, this method involves transforming primary human cells containing a human VRP-encoding gene with a construct (*i.e.*, vector) comprising an amplifiable gene [such as dihydrofolate reductase (DHFR) or others discussed below] and at least one flanking region of a length of at least about 150 bp that is homologous with a DNA sequence at the locus of the coding region of the VRP gene to provide amplification of the VRP gene.

The amplifiable gene must be at a site that does not interfere with expression of the VRP gene. The transformation is conducted such that the construct becomes homologously integrated into the genome of the primary cells to define an amplifiable region.

Primary cells comprising the construct are then selected for by means of the amplifiable gene or other marker present in the construct. The presence of the marker gene establishes the presence and integration of the construct into the host genome. No further selection of the primary cells need be made, since selection will be made in the second host. If desired, the occurrence of the homologous recombination event can be determined by employing PCR and either sequencing the resulting amplified DNA sequences or determining the appropriate length of the PCR fragment when DNA from correct homologous integrants is present and expanding only those cells containing such fragments. Also if desired, the selected cells may be amplified at this point by stressing the cells with the appropriate amplifying agent (such as methotrexate if the amplifiable gene is DHFR), so that multiple copies of the target gene are obtained. Preferably, however, the amplification step is not conducted until after the second transformation described below.

After the selection step, DNA portions of the genome, sufficiently large to include the entire amplifiable region, are isolated from the selected primary cells. Secondary mammalian expression host cells are then transformed with these genomic DNA portions and cloned, and clones are selected that contain the amplifiable region. The amplifiable region is then amplified by means of an amplifying agent if not already amplified in the primary cells. Finally, the secondary expression host cells now comprising multiple copies of the amplifiable region containing VRP are grown so as to express the gene and produce the protein.

A. Isolation of DNA Encoding VRP

The DNA encoding VRP may be obtained from any cDNA library prepared from tissue believed to possess the VRP mRNA and to express it at a detectable level. Accordingly, human VRP DNA can be conveniently obtained from a cDNA library prepared from human brain tissue, e.g., a glial cell line. The VRP-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries are screened with probes (such as antibodies to the VRP or oligonucleotides of about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding VRP is to use PCR methodology as described in section 14 of Sambrook *et al.*, *supra*.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various human tissues, preferably brain cell lines. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ³²P-labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

In some preferred embodiments, the nucleic acid sequence includes the native VRP signal sequence. Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries

using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, *supra*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Amino acid sequence variants of VRP are prepared by introducing appropriate nucleotide changes into the VRP DNA, or by synthesis of the desired VRP polypeptide. Such variants represent insertions, substitutions, and/or deletions of, residues within or at one or both of the ends of the amino acid sequence shown for the VRP in Figure 1. Preferably, these variants represent insertions and/or substitutions within or at one or both ends of the mature sequence, and/or insertions, substitutions and/or deletions within or at one or both of the ends of the signal sequence for VRP shown in Fig. 1. Any combination of insertion, substitution, and/or deletion is made to arrive at the final construct, provided that the final construct possesses the desired biological activity as defined herein. The amino acid changes also may alter post-translational processes of the VRP, such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intracellular location of the VRP by inserting, deleting, or otherwise affecting the leader sequence of the VRP.

Variations in the native sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. See also, for example, Table I therein and the discussion surrounding this table for guidance on selecting amino acids to change, add, or delete.

B. Insertion of Nucleic Acid into Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding native or variant VRP is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

The VRPs of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the VRP DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native VRP signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Pat. No. 5,010,182 issued 23 April 1991), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (e.g., the VRP presequence that normally directs secretion of VRP from human cells

in vivo) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal VRPs, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the mature VRP.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of VRP DNA. However, the recovery of genomic DNA encoding VRP is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the VRP DNA.

(iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet., 1:327 [1982]), mycophenolic acid (Mulligan *et al.*, Science, 209:1422 [1980]) or hygromycin. Sugden *et al.*, Mol. Cell. Biol., 5:410-413 (1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the VRP nucleic acid, such as DHFR or thymidine kinase. The

mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes VRP.

5 Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of VRP are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

10 For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the

15 synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding VRP. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells [particularly wild-type hosts that contain endogenous DHFR] transformed or

20 co-transformed with DNA sequences encoding VRP, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7.

25 Stinchcomb *et al.*, Nature, 282:39 (1979); Kingsman *et al.*, Gene, 7:141 (1979); Tschemper *et al.*, Gene, 10:157 (1980). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, Genetics, 85:12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are

30 complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi *et al.*, Curr. Genet., 12:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin

35 by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, Bio/Technology, 9:968-975 (1991).

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the VRP nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and

translation of particular nucleic acid sequence, such as the VRP nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to VRP-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native VRP promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the VRP DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of VRP as compared to the native VRP promoter.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems (Chang *et al.*, Nature, 275:615 [1978]; Goeddel *et al.*, Nature, 281:544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8:4057 [1980]; EP 36,776), and hybrid promoters such as the tac promoter. deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80:21-25 (1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding VRP (Siebenlist *et al.*, Cell, 20:269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding VRP.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem., 255:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7:149 [1968]; Holland, Biochemistry, 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

VRP transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a

retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the VRP sequence, provided such promoters are compatible with the host cell systems.

- 5 The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature, **273**:113 (1978); Mulligan and Berg, Science, **209**:1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA, **78**:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene, **18**:355-360 (1982). A system for expressing DNA in mammalian hosts using
10 the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Gray *et al.*, Nature, **295**:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, Nature, **297**:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA, **79**:5166-5170 (1982) on expression of the human interferon
15 β 1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, Proc. Natl. Acad. Sci. USA, **79**:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

- 20 Transcription of a DNA encoding the VRP of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, Proc. Natl. Acad. Sci. USA, **78**:993 [1981]) and 3' (Lusky *et al.*, Mol. Cell Bio., **3**:1108 [1983]) to the transcription unit, within an intron (Banerji *et al.*, Cell,
25 **33**:729 [1983]), as well as within the coding sequence itself. Osborne *et al.*, Mol. Cell Bio., **4**:1293 (1984). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv,
30 Nature, **297**:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the VRP-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

- 35 Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding VRP.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res., 9:309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding VRP. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of VRP that are biologically active VRP.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of VRP in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293:620-625 (1981); Mantei *et al.*, Nature, 281:40-46 (1979); EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of VRP is pRK5 (EP 307,247) or pSV16B. WO 91/08291 published 13 June 1991.

C. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is a particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 27C7. The complete genotype of 27C7 is *tonA Δ ptr3 phoA Δ E15 Δ (argF-lac)169 ompT Δ degP41 karF*. Strain 27C7 was deposited on 30 October 1991 in the American Type Culture Collection as ATCC No. 55,244. Alternatively, the strain of *E. coli* having mutant

periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990 may be employed. Alternatively still, methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for VRP-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290:140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer *et al.*, *supra*) such as, e.g., *K. lactis* [MW98-8C, CBS683, CBS4574; Louvencourt *et al.*, J. Bacteriol., 737 (1983)], *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilae* (ATCC 36,906; Van den Berg *et al.*, *supra*), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* [EP 402,226]; *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol., 28:265-278 [1988]); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA, 76:5259-5263 [1979]); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolyposcladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun., 112:284-289 [1983]; Tilburn *et al.*, Gene, 26:205-221 [1983]; Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81:1470-1474 [1984]) and *A. niger*. Kelly and Hynes, EMBO J., 4:475-479 (1985).

Suitable host cells for the expression of glycosylated VRP are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, e.g., Luckow *et al.*, Bio/Technology, 6:47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, Nature, 315:592-594 (1985). A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the VRP-encoding DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the VRP is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the VRP-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen., 1:561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol., 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 [1980]); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for VRP production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact., 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, *etc.*, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185:527-537 (1990) and Mansour *et al.*, Nature, 336:348-352 (1988).

D. Culturing the Host Cells

Prokaryotic cells used to produce the VRP polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the VRP of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz. 58:44 (1979), Barnes and Sato, Anal. Biochem. 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GentamycinTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

E. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75:734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native VRP polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

5 **F. Purification of VRP Polypeptide**

VRP preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the VRP is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100)

When VRP is produced in a recombinant cell other than one of human origin, the VRP is completely
10 free of proteins or polypeptides of human origin. However, it is necessary to purify VRP from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to VRP. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. VRP thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC;
15 chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

In the preferred embodiment, the Flt4 receptor-IgG fusion is immobilized on an affinity chromatography column and the VRP can be isolated by affinity purification using this column. Alternatively, the VRP is joined
20 at its N-terminus to a glycoprotein D sequence and is passed through an affinity chromatography column on which is immobilized an anti-gD monoclonal antibody such as 5B6, which is specific for a glycoprotein D sequence.

VRP variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native VRP, taking account of any substantial changes in properties occasioned by the variation. For
25 example, preparation of a VRP fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal anti-VRP column can be employed to absorb the VRP variant by binding it to at least one remaining immune epitope.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit
30 proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native VRP may require modification to account for changes in the character of VRP or its variants upon expression in recombinant cell culture.

G. Covalent Modifications of VRP Polypeptides

35 Covalent modifications of VRP polypeptides are included within the scope of this invention. Both native VRP and amino acid sequence variants of the VRP may be covalently modified. One type of covalent modification of the VRP is introduced into the molecule by reacting targeted amino acid residues of the VRP with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the VRP.

Cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazolyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed under alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as with the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking VRP to a water-insoluble support matrix or surface for use in the method for purifying anti-VRP antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutamyl and asparagyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the VRP polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native VRP, and/or adding one or more glycosylation sites that are not present in the native VRP.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the VRP polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native VRP sequence (for O-linked glycosylation sites). For ease, the VRP amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the VRP polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, *supra*.

Another means of increasing the number of carbohydrate moieties on the VRP polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the VRP polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al.*, Arch. Biochem. Biophys., 252:52 (1987)

and by Edge *et al.*, Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, Meth. Enzymol., 138:350 (1987).

5 Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.*, J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of VRP comprises linking the VRP polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

10 Since it is often difficult to predict in advance the characteristics of a variant VRP, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. A change in the immunological character of the VRP molecule, such as affinity for a given antibody, is also able to be measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its mitogenic activity by comparison to the activity observed for native VRP in the same assay. For example,
15 one can screen for the ability of the variant VRP to stimulate protein kinase activity of the Flt4 receptor as described in Example 5 herein. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

H. Epitope-tagged VRP

20 This invention encompasses chimeric polypeptides comprising VRP fused to another polypeptide. In one preferred embodiment, the chimeric polypeptide comprises a fusion of the VRP with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the VRP. Such epitope-tagged forms of the VRP are desirable, as the presence thereof can be detected using a labeled antibody against the tag polypeptide. Also, provision of the epitope tag
25 enables the VRP to be readily purified by affinity purification using the anti-tag antibody. Affinity purification techniques and diagnostic assays involving antibodies are described later herein.

Tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al.*, Mol. Cell. Biol., 8:2159-2165 [1988]); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, Molecular and Cellular Biology,
30 5:3610-3616 [1985]); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody. Paborsky *et al.*, Protein Engineering, 3(6):547-553 (1990). Other tag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp *et al.*, BioTechnology, 6:1204-1210 [1988]); the KT3 epitope peptide (Martin *et al.*, Science, 255:192-194 [1992]); an α -tubulin epitope peptide (Skinner *et al.*, J. Biol. Chem., 266:15163-15166 [1991]); and the T7 gene 10 protein peptide tag. Lutz-Freyermuth *et al.*, Proc. Natl. Acad. Sci. USA, 87:6393-
35 6397 (1990). Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

The general methods suitable for the construction and production of epitope-tagged VRP are the same as those disclosed hereinabove with regard to (native or variant) VRP. VRP-tag polypeptide fusions are most conveniently constructed by fusing the cDNA sequence encoding the VRP portion in-frame to the tag

polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the VRP-tag polypeptide chimeras of the present invention, nucleic acid encoding the VRP will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible.

5 Epitope-tagged VRP can be conveniently purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached is most often agarose, but other matrices are available (e.g. controlled pore glass or poly(styrenedivinyl)benzene). The epitope-tagged VRP can be eluted from the affinity column by varying the buffer pH or ionic strength or adding chaotropic agents, for example.

2. Therapeutic Uses, Compositions, and Administration of VRP

10 VRP is believed to find therapeutic use for treating mammals via stimulation or inhibition of growth and/or differentiation and/or activation of cells having the Flt4 receptor or one or more other VRP receptors. Exogenous VRP may be administered to a patient in these circumstances. The human VRP is clearly useful insofar as it can be administered to a human having depressed levels of endogenous VRP, preferably in the situation where such depressed levels lead to a pathological disorder, or where there is lack of activation or
15 inhibition of the Flt4 receptor or one or more other VRP receptors.

Various potential therapeutic uses of VRP include those in which VEGF is useful. Examples of these include uses associated with the vascular endothelium, such as the treatment of traumata to the vascular network, in view of the demonstrated rapid promotion by VEGF of the proliferation of vascular endothelial cells that would surround the traumata and in view of the relationship between VEGF and the VRP established herein.

20 Examples of such traumata that could be so treated include, but are not limited to, surgical incisions, particularly those involving the heart, wounds, including lacerations, incisions, and penetrations of blood vessels, and surface ulcers involving the vascular endothelium such as diabetic, haemophiliac, and varicose ulcers. Other physiological conditions that could be improved based on the selective mitogenic character of the VRP are also included herein.

25 For the traumatic indications referred to above, the VRP molecule will be formulated and dosed in a fashion consistent with good medical practice taking into account the specific disorder to be treated, the condition of the individual patient, the site of delivery of the VRP, the method of administration, and other factors known to practitioners.

30 Additional indications for the VRP are in the treatment of full-thickness wounds such as dermal ulcers, including the categories of pressure sores, venous ulcers, and diabetic ulcers, as well as of full-thickness burns and injuries where angiogenesis is required to prepare the burn or injured site for a skin graft or flap. In this case the VRP is either applied directly to the site or it is used to soak the skin or flap that is being transplanted prior to grafting. In a similar fashion, the VRP can be used in plastic surgery when reconstruction is required following a burn or other trauma, or for cosmetic purposes.

35 Angiogenesis is also important in keeping wounds clean and non-infected. The VRP can therefore be used in association with general surgery and following the repair of cuts and lacerations. It is particularly useful in the treatment of abdominal wounds with a high risk of infection. Neovascularization is also key to fracture repair, since blood vessels develop at the site of bone injury. Administration of the VRP to the site of a fracture is therefore another utility.

In cases where the VRP is being used for topical wound healing, as described above, it may be administered by any of the routes described below for the re-endothelialization of vascular tissue, or more preferably by topical means. In these cases, it will be administered as either a solution, spray, gel, cream, ointment, or dry powder directly to the site of injury. Slow-release devices directing the VRP to the injured site will also be used. In topical applications, the VRP will be applied at a concentration ranging from about 50 to 1,000 µg/mL, either in a single application, or in dosing regimens that are daily or every few days for a period of one week to several weeks. Generally, the amount of topical formulation administered is that which is sufficient to apply from about 0.1 to 100 µg/cm² of the VRP, based on the surface area of the wound.

The VRP can be used as a post-operative wound healing agent in balloon angioplasty, a procedure in which vascular endothelial cells are removed or damaged, together with compression of atherosclerotic plaques. The VRP can be applied to inner vascular surfaces by systemic or local intravenous application either as intravenous bolus injection or infusions. If desired, the VRP can be administered over time using a micrometering pump. Suitable compositions for intravenous administration comprise the VRP in an amount effective to promote endothelial cell growth and a parenteral carrier material. The VRP can be present in the composition over a wide range of concentrations, for example, from about 50 µg/mL to about 1,000 µg/mL using injections of 3 to 10 mL per patient, administered once or in dosing regimens that allow for multiple applications. Any of the known parenteral carrier vehicles can be used, such as normal saline or 5-10% dextrose.

The VRP can also be used to promote endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted vessels or synthetic material, for example, the VRP can be applied to the surfaces of the graft and/or at the junctions of the graft and the existing vasculature to promote the growth of vascular endothelial cells. For such applications, the VRP can be applied intravenously as described above for balloon angioplasty or it can be applied directly to the surfaces of the graft and/or the existing vasculature either before or during surgery. In such cases it may be desired to apply the VRP in a thickened carrier material so that it will adhere to the affected surface. Suitable carrier materials include, for example, 1-5% carbopol. The VRP can be present in the carrier over a wide range of concentrations, for example, from about 50 µg/mg to about 1,000 µg/mg. Alternatively, the VRP can be delivered to the site by a micrometering pump as a parenteral solution.

The VRP can also be employed to repair vascular damage following myocardial infarction and to circumvent the need for coronary bypass surgery by stimulating the growth of a collateral circulation. The VRP is administered intravenously for this purpose, either in individual injections or by micrometering pump over a period of time as described above or by direct infusion or injection to the site of damaged cardiac muscle.

Therapeutic formulations of VRP are prepared for storage by mixing VRP having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., [1980]), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose,

mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

5 The VRP also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacrylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

10 VRP to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. VRP ordinarily will be stored in lyophilized form or in solution.

Therapeutic VRP compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

15 The route of VRP administration is in accord with known methods, *e.g.*, those routes set forth above for specific indications, as well as the general routes of injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional means, or sustained release systems as noted below. VRP is administered continuously by infusion or by bolus injection. Generally, where the disorder permits, one should formulate and dose the VRP for site-specific delivery. This is convenient in the case of wounds and ulcers.

20 Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res., 15:167-277 (1981) and Langer, Chem. Tech., 12:98-105 (1982) or poly(vinylalcohol)], polylactides (U.S. Patent No. 3,773,919, EP 58,481), 25 copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22:547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron DepotTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

30 While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, 35 stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release VRP compositions also include liposomally entrapped VRP. Liposomes containing VRP are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82:3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77:4030-4034 (1980); EP 52,322; EP 36,676; EP

88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal VRP therapy.

5 When applied topically, the VRP is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal
10 patches, plasters, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the VRP formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as PEG to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and
15 alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullulan; agarose; carrageenan; dextrans; dextrans; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The
20 preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the VRP held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is
25 methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight PEGs to obtain the proper viscosity. For example, a mixture of a PEG of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term "water soluble" as applied to the polysaccharides and PEGs is meant to include colloidal
30 solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

35 If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the VRP is present in an amount of about 300-1000 mg per ml of gel.

An effective amount of VRP to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the

optimal therapeutic effect. Typically, the clinician will administer the VRP until a dosage is reached that achieves the desired effect. A typical daily dosage for systemic treatment might range from about 1 µg/kg to up to 10 mg/kg or more, depending on the factors mentioned above. As an alternative general proposition, the VRP is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a VRP level greater than about 0.1 ng/cc up to a maximum dose that is efficacious but not unduly toxic. This intra-tissue concentration should be maintained if possible by continuous infusion, sustained release, topical application, or injection at empirically determined frequencies. The progress of this therapy is easily monitored by conventional assays.

It is within the scope hereof to combine the VRP therapy with other novel or conventional therapies (e.g., growth factors such as VEGF, acidic or basic fibroblast growth factor (aFGF or bFGF, respectively), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I or IGF-II), nerve growth factor (NGF), anabolic steroids, EGF or TGF-α) for enhancing the activity of any of the growth factors, including the VRP, in promoting cell proliferation and repair. It is not necessary that such co-treatment drugs be included *per se* in the compositions of this invention, although this will be convenient where such drugs are proteinaceous. Such admixtures are suitably administered in the same manner and for the same purposes as the VRP used alone. The useful molar ratio of VRP to such secondary growth factors is typically 1:0.1-10, with about equimolar amounts being preferred.

3. Non-Therapeutic, Diagnostic Uses for VRP

The nucleic acid encoding the VRP may be used as a diagnostic for tissue-specific typing. For example, such procedures as *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding VRP is present in the cell type(s) being evaluated. VRP nucleic acid or polypeptide may also be used as diagnostic markers. For example, the VRP may be labeled, using the techniques described herein, and expression of nucleic acid molecules encoding a Flt4 receptor or another VRP receptor can be quantified, using the labelled VRP.

If the human VRP-encoding nucleic acid is localized to a human chromosome, the nucleic acid for human VRP can be used as a marker for this human chromosome.

VRP nucleic acid is also useful for the preparation of VRP polypeptide by recombinant techniques exemplified herein.

Isolated VRP polypeptide may be used in quantitative diagnostic assays as a standard or control against which samples containing unknown quantities of VRP may be prepared.

VRP preparations are also useful in generating antibodies, as standards in assays for VRP (e.g., by labeling VRP for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), for detecting the presence of the Flt4 receptor or one or more other VRP receptors in a biological sample (e.g., using a labeled VRP), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, or the like.

The VRP is also useful as a diagnostic tool. For example, the VRP can be produced in prokaryotic cells using the techniques elaborated herein and the unglycosylated protein so produced can be used as a molecular weight marker. The deduced molecular weight (mw) of the VRP is about 44.8 kDa. To use the VRP as a molecular weight marker, gel filtration chromatography or SDS-PAGE, for example, will be used to separate

protein(s) for which it is desired to determine their molecular weight(s) in substantially the normal way. The VRP and other molecular weight markers will be used as standards to provide a range of molecular weights. For example, phosphorylase b (mw = 97,400), bovine serum albumin (mw = 68,000), ovalbumin (mw = 46,000), VRP (mw = 44,800), trypsin inhibitor (mw = 20,100), and lysozyme (mw = 14,400) can be used as mw markers.

5 The other molecular weight markers mentioned here can be purchased commercially from Amersham Corporation, Arlington Heights, IL, for example. Often, the molecular weight markers will be labeled to enable easy detection following separation. Techniques for labeling antibodies and proteins are discussed herein and are well known in the art. For example, the molecular weight markers may be biotinylated and, following separation on SDS-PAGE, for example, the blot can be incubated with streptavidin-horseradish peroxidase. The

10 bands can then be detected by light detection.

It may also be useful to grow certain cells having the Flt4 receptor or one or more other VRP receptors *ex vivo* using the VRP as an angiogenic factor or growth factor. Thus, for example, the VRP can be used as a growth factor in the *in vitro* culturing of endothelial cells. For such uses, the VRP can be added to the cell culture medium at a concentration from about 10 pg/mL to about 10 ng/mL.

15 These cells which are to be grown *ex vivo* may simultaneously be exposed to other known growth factors or cytokines. Exemplary cytokines include the interleukins (*e.g.*, IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (Epo), lymphotoxin, steel factor (SLF), tumor necrosis factor (TNF), and gamma-interferon. This results in proliferation

20 and/or differentiation of the cells having the Flt4 receptor or one or more other VRP receptors.

In yet another aspect of the invention, the VRP may be used for affinity purification of the Flt4 receptor or one or more other VRP receptors. Briefly, this technique involves covalently attaching the VRP to an inert and porous matrix (*e.g.*, agarose reacted with cyanogen bromide). A solution containing the Flt4 receptor or other VRP receptor(s) can then be passed through the chromatographic material and can be subsequently released

25 by changing the elution conditions (*e.g.* by changing pH or ionic strength).

The purified VRP, and the nucleic acid encoding it, may also be sold as reagents for mechanism studies of VRP and its cognate receptors, to study the role of the VRP and the Flt4 receptor or other VRP receptors in normal growth and development, as well as abnormal growth and development, *e.g.* in malignancies.

The VRP may be used for competitive screening of potential agonists or antagonists for binding to the

30 Flt4 receptor or other VRP receptors. VRP variants are useful as standards or controls in assays for the VRP, provided that they are recognized by the analytical system employed, *e.g.* an anti-VRP antibody.

4. VRP Antibody Preparation

A description follows as to the production of exemplary antibodies as defined herein. These exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, or heteroconjugate antibodies.

35 A. Polyclonal Antibodies

Polyclonal antibodies to the VRP generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the VRP and an adjuvant. It may be useful to conjugate the VRP to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example

maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glytaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

5 Animals are immunized against the immunogenic conjugates or derivatives by combining 1 mg of 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freud's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freud's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for anti-VRP antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with a conjugate of the same VRP with a different protein and/or
10 the conjugation is through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

B. Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring
15 mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the anti-VRP monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods. U.S. Pat. No. 4,816,567.

20 In the hybridoma method, a mouse or other appropriate host animal, such as hamster, is immunized as hereinabove described to elicit lymphocytes that produce, or are capable of producing, antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as PEG, to form a hybridoma cell. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986).

25 The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

30 Preferred myeloma cells are those that fuse efficiently, support stable high-level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and
35 mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. Kozbor, J. Immunol., 133:3001 (1984); Brodeur *et al.*, Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987). See, also, Boerner *et al.*, J. Immunol., 147:86-95 (1991) and WO 91/17769, published 28 November 1991, for techniques for the production of human monoclonal antibodies.

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against VRP. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

5 The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-104 (Academic Press, 1986). Suitable culture media for
10 this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

15 Alternatively, it is now possible to produce transgenic animals (*e.g.* mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will
20 result in the production of human antibodies upon antigen challenge. See, *e.g.* Jakobovits *et al.*, Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits *et al.*, Nature, 362:255-258 (1993).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, Nature, 348:552-554 (1990), using the VRP to select for a suitable antibody or antibody fragment. Clackson *et al.*, Nature, 352:624-628 (1991) and Marks *et al.*, J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using
25 phage libraries. Subsequent publications describe the production of high-affinity (nM range) human antibodies by chain shuffling (Mark *et al.*, Bio/Technol., 10:779-783 [1992]), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries. Waterhouse *et al.*, Nuc. Acids Res., 21:2265-2266 (1993). Thus, these techniques are viable alternatives to traditional monoclonal antibody
30 hybridoma techniques for isolation of "monoclonal" antibodies (especially human antibodies) that are encompassed by the present invention.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a
35 preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (Morrison *et al.*,

Proc. Nat. Acad. Sci. USA, 81, 6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-VRP monoclonal antibody herein.

Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an VRP and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

For diagnostic applications, the antibodies of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter *et al.*, Nature, 144:945 (1962); David *et al.*, Biochemistry, 13:1014 (1974); Pain *et al.*, J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be a VRP, or an immunologically reactive portion thereof) to compete with the test sample analyte (VRP) for binding with a limited amount of antibody. The amount of VRP in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected (VRP). In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, e.g., US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

C. Humanized Antibodies

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, Nature, 321:522-525 [1986]; Riechmann *et al.*, Nature, 332:323-327 [1988]; Verhoeven *et al.*, Science, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567, *supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues, and possibly some FR residues, are substituted by residues from analogous sites in rodent antibodies.

It is important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding. For further details see WO 92/22653, published 23 December 1992.

D. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the VRP, the other one is for any other antigen, and preferably for a receptor or receptor subunit. For example, bispecific antibodies specifically binding the Flt4 receptor and the VRP are within the scope of the present invention.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities. Millstein and Cuello, Nature, 305:537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker *et al.*, EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences.

The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, Methods in Enzymology, 121:210 (1986).

E. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection. WO 91/00360; WO 92/200373; EP 03089. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed, for example, in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

25 5. Uses of VRP Antibodies

i. Therapeutic Uses

VRP antibodies may be useful in certain therapeutic indications to block activity of the VRP (for example, to block excess activation or inhibition of the Flt4 receptor or another receptor that binds to VRP, and to block neovascularization, re-endothelialization, and angiogenesis). Specifically, the VRP antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate

carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VRP antibodies of the present invention are expected to be especially useful in reducing the severity of AMD.

For therapeutic applications, the VRP antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The VRP antibodies also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes or to the lymph, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

Such dosage forms encompass pharmaceutically acceptable carriers that are inherently non-toxic and non-therapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and PEG. Carriers for topical or gel-based forms of VRP antibodies include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, PEG, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations. The VRP antibody will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the VRP antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *supra* and Langer, *supra*, or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *supra*), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron DepotTM (injectable micropheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl

acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated VRP antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release VRP antibody compositions also include liposomally entrapped antibodies.

10 Liposomes containing the VRP antibodies are prepared by methods known in the art, such as described in Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Ordinarily, the liposomes are the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal VRP antibody therapy. Liposomes with enhanced circulation time are disclosed

15 in U.S. Patent No. 5,013,556.

Another use of the present invention comprises incorporating VRP antibodies into formed articles. Such articles can be used in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles.

For the prevention or treatment of disease, the appropriate dosage of VRP antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibodies are administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the VRP antibody, and the discretion of the attending physician. The VRP antibody is suitably administered to the patient at one time or over a series of treatments.

20

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg of VRP antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

25

30

According to another embodiment of the invention, the effectiveness of the VRP antibody in preventing or treating disease may be improved by administering the VRP antibody serially or in combination with another proteinaceous agent that is effective for those purposes, such as those enumerated below, or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the VRP antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

35

In one embodiment, vascularization of tumors is attacked in combination therapy. One or more VRP antibodies are administered to tumor-bearing patients at therapeutically effective doses as determined, for example, by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then the proteinaceous auxiliary agent is administered, alone or in combination with another auxiliary agent. Such agents include, e.g., TNF, an antibody capable of inhibiting or neutralizing the angiogenic activity of aFGF or bFGF or hepatocyte growth factor (HGF), an rhVEGF antagonist as described in WO 94/10202, *supra*, alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), GM-CSF, or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (WO 91/01753 published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of VRP antibody and TNF and/or other auxiliary agent is repeated until the desired clinical effect is achieved. Alternatively, the VRP antibody or antibodies are administered together with TNF and, optionally, other auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the VRP antibody. Treatment with VRP antibodies optimally may be suspended during periods of wound healing or desirable neovascularization.

ii. Other Uses

The VRP antibodies of the invention also are useful as affinity purification agents. In this process, the antibodies against VRP are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the VRP to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VRP, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VRP from the antibody.

VRP antibodies may also be useful in diagnostic assays for VRP, e.g., detecting its expression in specific cells, tissues, or serum. The antibodies are labeled in the same fashion as VRP described above and/or are immobilized on an insoluble matrix. VRP antibodies also are useful for the affinity purification of VRP from recombinant cell culture or natural sources. VRP antibodies that do not detectably cross-react with other proteins can be used to purify VRP free from these other known proteins. Suitable diagnostic assays for VRP and its antibodies are described above.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

EXAMPLE 1

Isolation of cDNA Clones Encoding Human Flt4 Receptor

cDNA synthesized from mRNA purified from the human megakaryocyte leukemia cell line CMK11-5 was amplified with redundant PCR primers based on the conserved regions of tyrosine kinase receptors. Wilks, *Proc. Natl. Acad. Sci. USA*, **86**: 1603-1607 (1989). One amplified fragment of about 180 bp with a unique DNA sequence (designated SAL-S1 or tk1; PCT/US93/00586, *supra*) was used to screen (Janssen *et al.*, *supra*) cDNA libraries from CMK11-5 and DAMI cells to obtain overlapping clones that encoded the full-length short form of Flt4 receptor (1298 amino acids). The sequence of the assembled Flt4-encoding clones matched that reported from an anerythroleukemia cell line (Pajusola *et al.*, *Cancer Res.*, *supra*); it encodes 8 amino acid differences from another reported Flt4 sequence. Galland *et al.*, *Oncogene*, *supra*. Clones encoding the long form of Flt4 (1363 amino acids) were constructed by synthesizing the differing 3' DNA sequence of about 200 bp based on the published sequence. Pajusola *et al.*, *Oncogene*, **8**, *supra*.

EXAMPLE 2

Receptor IgG Fusion Proteins, Flt4/IgG Antiserum,

and G61 FACS Analysis

Flt1/IgG (Park *et al.*, *supra*), Flk1/IgG (Park *et al.*, *supra*), Rse/IgG (Godowski *et al.*, *Cell*, **82**: 355-358 [1995]), and Htk/IgG (Bennett *et al.*, *J. Biol. Chem.*, **269**: 14211-14218 [1994]) were produced as described in these references. For Flt4/IgG, DNA encoding the extracellular domain of the Flt4 receptor (amino acids 1-775) was spliced to the Fc region of a human IgG heavy chain at the unique BstEII site in the plasmid pBSSK-Fc (pBSSK-CH2CH3). Bennett *et al.*, *J. Biol. Chem.*, **266**: 23060-23067 (1991). The open reading frame encoding Flt4/IgG was cloned in the mammalian expression vector pRK5 (Suva *et al.*, *Science*, **237**: 893-896 [1987]) to yield the plasmid pRK5.tk1.lg1.1. This plasmid was transfected by electroporation (Janssen *et al.*, *supra*) into 293 cells (ATCC CRL 1651), and after 3-4 days, Flt4/IgG was purified from the serum-free conditioned medium with protein A agarose (Calbiochem). Flt4 antiserum was generated by injection of purified Flt4/IgG into rabbits.

By using this fusion protein to screen cell lines for membrane-bound VRP by FACS analysis, one positive cell line was identified, G61, described below.

The human glioma cell line, G61 (Hamel *et al.*, *J. Neurosci. Res.*, **34**: 147-157 [1993]), was cultured in F12:DMEM (50:50) (high glucose) containing 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. For FACS analysis of Flt4/IgG binding to G61 cells, 1 million cells were incubated with 70 nM receptor-IgG fusion protein in phosphate-buffered saline (PBS), 5% goat serum, 2% rabbit serum for 60 min. at 4°C and then stained with 10 µg/mL biotin-SP-conjugated goat anti-human Fc antibody and 10 µg/mL R-phycoerythrin-conjugated streptavidin (Jackson Immuno Research). G61 caused about a 10-fold shift in peak fluorescence intensity that was specific for Flt4/IgG as compared to Rse/IgG, an unrelated tyrosine kinase receptor complex (Fig. 2). Attempts to expression clone this putative membrane-bound VRP by the transfection of pools of cDNA clones into COS cells followed by screening with labeled Flt4/IgG yielded no positives from 640 pools of 1000-5000 clones each.

EXAMPLE 3

Isolation of cDNA Clones Encoding Human VRP

A cDNA library was prepared from polyA⁺ RNA isolated as described in Cathala *et al.*, DNA, 2: 329-335 (1983) and Aviv and Leder, Proc. Natl. Acad. Sci. USA, 69: 1408-1412 (1972) from the human glioma cell line, G61. Hamel *et al.*, *supra*. cDNA was prepared from this RNA with reagents from GIBCO/BRL (SuperScript) and cloned in the plasmid pRK5B (Holmes *et al.*, Science, 253: 1278-1280 [1991]) digested with XhoI and NotI. Clones encoding VRP were isolated by screening the cDNA library with synthetic oligonucleotide probes based on an EST sequence (GenBank locus HSC1WF111), which showed a reasonable match to VEGF. The EST sequence of HSC1WF111 is 299 bp and is 36% identical to VEGF over 50 residues, including an 11 of 13 residue match beginning at VEGF amino acid 56. The sequence is as follows:

5'-CCGTCTACAGATGTGGGGGTTGCTGCAATAGTGAGGGGCTGCAGTGCATGAACACCAGCAGCAG
AGCTACCTCAGNAAGACGTTATTTGAAATTACAGTGCCTCTCTCTCAAGGCCCAAAACCAGTAA
CAATCAGTTTGGCAATCACACTTCCTGCCGATGCATGTCTAAACTGGATGTTTACAGACAAGT
TCATTCCATTATTAGACGTTCCCTGCCAGCAACACTACCACAGTGTGAGGCAGCGAACAAGACC
TGCCCCACCAATTACATGTGGAATAATCACATCTGCAGATGCCTG (SEQ ID NO: 6).

The sequences of the oligonucleotide probes ovh1.4 and ovh1.5 employed are indicated below.

ovh1.4: 5'-CTGGTGTTTCATGCACTGCAGCCCCTCACTATTGCAGCAACCCCCACATCT (SEQ ID NO: 7)

ovh1.5: 5'-GCATCTGCAGATGTGATTATCCACATGTAATTGGTGGGGCAGGTCTTGT (SEQ ID NO: 8)

These two probes were ³²P labeled and hybridized in 20% formamide at 42°C with a final wash in 30 mM NaCl/3 mM trisodium citrate at 55°C. Janssen, Current Protocols in Molecular Biology, John Wiley & Sons (1995). Seven positives were identified and characterized from 650,000 clones screened. The positives fell into three groups by restriction mapping and DNA sequencing.

Clones VH1.4 (pRK.vh1.4.1) and VH1.6 included the full coding region (Fig. 3A) and were sequenced completely. They differ only in length and the lack of two T's preceeding the 3' poly A sequence in VH1.6. Clone VH1.2 is collinear with VH1.4. Clones VH1.3, VH1.5, and VH1.7 are identical and have a 557 bp deletion when compared with VH1.4 (a deletion of bp 519-1075), and clone VH1.1 has a 152 bp deletion when compared with VH1.4 (a deletion of bp 924-1075). The nucleotide and deduced amino acid sequences of VH1.4 are shown in Figure 1.

The sequence contained an open reading frame of 419 amino acids beginning with an ATG codon preceded by a purine residue at position -3 as expected for a translation initiation site. Kozak, Nucl. Acids Res., 12: 857-872 (1984). About 250 bp 5' of this ATG are two in-frame ATG codons followed shortly (4 or 10 amino acids) by a stop codon. Both of these ATG's have a pyrimidine at position -3 and would not be expected to function as a strong translation initiation site. Kozak, *supra*. The encoded amino acid sequence immediately following the start of the 419 amino acid reading frame is hydrophobic, indicative of an amino-terminal secretion signal sequence. Perlman and Halvorson, J. Mol. Biol., 167: 391-409 (1983). See Figure 3A. The most likely cleavage site for this sequence would be after amino acid 20, although cleavage following residues 15 or 16

cannot be excluded. von Heijne, *Nucl. Acids Res.*, 14: 4683-4690 (1986). The open reading frame is preceded by a GC-rich 5' untranslated region of about 380 bp and followed by a 3' untranslated region of about 400 bp.

The predicted mature amino acid sequence of human VRP contains 399 amino acid residues (translated $M_r = 44.8$ kDa), of which 37 (9.3%) are cysteine residues; there are three potential N-linked glycosylation sites (Fig. 3A). An alignment of the amino acid sequence of VRP with the six forms of VEGF and PlGF shows that it is most similar to VEGF₁₂₁ (32% identical) and PlGF₁₃₁ (27% identical) (Fig. 3B); the locations of 8 of the 9 cysteine residues are conserved. While VRP does not contain the regions of basic amino acids found in some forms of VEGF and PlGF, it is considerably larger than VEGF and contains a cysteine-rich C-terminal half of the molecule that is not found in VEGF. This cysteine-rich domain has four copies of the pattern Cys followed by ten non-Cys residues followed by Cys-X followed by Cys-X and then by Cys (Fig. 3B), a repeat found more than 50 times in a diptran Balbiani ring 3 protein. Paulsson *et al.*, *J. Mol. Biol.*, 211: 331-349 (1990). Without being limited to any one theory, VRP may interact with other membrane-bound proteins on these cells via the cysteine residues; such an intermolecular interaction has been proposed for the Balbiani protein. Paulsson *et al.*, *supra*.

Two of the cDNA clones (VH1.1 and VH1.3) contained a 152 or 557 bp deletion when compared with VH1.4 (Fig. 3A). Both these deletions end at the same nucleotide and are presumed to be the result of alternative splicing. Both deletions would be expected to encode the same frame-shifted protein 3' of the deletion which terminates at a stop codon within 15 amino acids. The protein encoded by VH1.3 would include none of the core cysteine region similar with VEGF. VH1.1 contains much of the region that is similar to VEGF; its deletion, however, is not analogous to the various known forms of VEGF or PlGF. Ferrara *et al.*, *supra*; Maglione *et al.*, *supra*; Hauser and Weich, *supra*.

Figure 4 discloses an alignment of VH1.4 (top) with 11 EST cDNA sequences from GenBank. It is noted that the 3' EST's are at the polyA end and that the EST's cover only a little more than half of the full-length sequence of VH1.4.

EXAMPLE 4

Receptor IgG Precipitation of ³⁵S-Labeled VRP

To determine whether VRP is a ligand for Flt4, expression plasmids containing the VH1.4 cDNA clone, as well as control plasmids (the expression vector alone or with VEGF or PlGF DNA), were transfected into COS7 cells and the proteins labeled with ³⁵S amino acids. Conditioned media from these cells was precipitated with Flt4/IgG and Flk1/IgG. Specifically, the VRP expression plasmid, pRK.vh1.4.2, was constructed by deleting about 360 bp of 5' untranslated sequence (5' of the *Age*I site (Fig. 3A) from VH1.4). This DNA and control plasmids encoding VEGF₁₆₅ (Houck *et al.*, *Mol. Endocrinol.*, 5: 1806-1814 [1991]), PlGF₁₅₂ (Park *et al.*, *supra*), or the vector alone (pRK5; Suva *et al.*, *supra*) were transfected into COS7 cells with DEAE-dextran. Janssen *et al.*, *supra*. Two days after transfection, the cells were pulse-labeled in 10-cm dishes for 5 hours with 5 mL of methionine- and cysteine-free DMEM supplemented with 100 μ Ci/mL of ³⁵S amino acids (Pro-MixTM brand; Amersham #SJQ0079) at 37°C, and then chased with DMEM for 7 hours. The labeled conditioned medium was concentrated 10-fold by spin concentration (Centricon-10TM brand; Amicon #4203). Fifty μ L of the concentrated medium was incubated with 3 μ g of receptor IgG and 80 μ L of a 50% slurry of protein A agarose (Calbiochem) overnight at 4°C. The precipitates were washed with PBS/0.1% Triton X-100.

boiled in SDS sample buffer, and electrophoresed on 12% SDS polyacrylamide gels (Novex #EC60052). The gels were treated with autoradiography enhancer (duPont #NEF974) and exposed overnight at -70° C.

Two specific bands of 53 kDa and 33 kDa were precipitated from the VRP transfection by the Flt4/IgG; these bands were absent in the vector transfection. Little or no specific precipitation of these two bands was found with Flt1/IgG or Flk1/IgG. At times, some VRP precipitation was detected with Flk1/IgG, suggesting that VRP may have a low-affinity interaction with Flk1. Transfection with a VEGF-expressing plasmid showed the expected precipitation of a strong band of about 22 kDa with Flt1/IgG and Flk1/IgG (DeVries *et al.*, *supra*; Quinn *et al.*, *supra*; Millauer *et al.*, *supra*; Terman *et al.*, *Biochem. Biophys. Res. Commun.*, *supra*), but no precipitation with Flt4/IgG. Similar experiments with labeled PIGF showed no precipitation by Flt4/IgG, but did give the expected precipitation by Flt1/IgG, but not by Flk1/IgG. Park *et al.*, *supra*. These data indicate that the VRP binds to the extracellular domain of the Flt4 receptor, but does not interact (or does so much more weakly) with the VEGF receptors Flt1 or Flk1. They also confirm the lack of an interaction of VEGF with Flt4 (Pajusola *et al.*, *Oncogene*, 2, *supra*) and indicate that PIGF is also not a ligand for this receptor.

EXAMPLE 5

15 Tyrosine Phosphorylation of Flt4 Receptor

To assay Flt4 tyrosine phosphorylation (also described in PCT/US93/00586, *supra*), Flt4 was expressed in 293 cells and Flt4 phosphorylation monitored by phosphotyrosine immunoblot. Specifically, DNA encoding the long form of human Flt4 was cloned into the mammalian expression vector pRK5 (Suva *et al.*, *supra*) to give the plasmid pRK.tk1-3.1. This plasmid was co-transfected with a plasmid containing a miroglycoside phosphotransferase (neo) transcription unit into 293 cells by calcium phosphate precipitation (Janssen *et al.*, *supra*), and stably transfected lines were selected by growth on G418 (Gibco). One clonal cell line expressing Flt4 (clone 31), as determined by FACS analysis with Flt4/IgG antiserum, and untransfected 293 cells were used in the Flt4 tyrosine phosphorylation assays. One million cells in 100 μ L of PBS/0.1% bovine serum albumin (BSA) were mixed with 100 μ L of sample and incubated at 37° C for 15 minutes. The cells were then collected by centrifugation and lysed in 250 μ L of 0.15 M NaCl, 10% glycerol, 1% Triton X-100, 50 mM HEPES pH 7.3, 4 μ g/mL PMSF, 0.02 u/mL aprotinin (Sigma A6279), and 20 mM sodium orthovanadate. Flt4 was immunoprecipitated by the addition of 8 μ L of rabbit Flt4/IgG antiserum and 30 μ L of protein A agarose. Washed precipitates were boiled in SDS sample buffer, electrophoresed on polyacrylamide gels (Novex), transferred to nitrocellulose (Janssen *et al.*, *supra*), and probed with an anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology) and an alkaline phosphatase detection system (Promega).

Samples containing VRP or VEGF were prepared by the electroporation of expression plasmids encoding VH1.4 (pRK.vh1.4.2) or VEGF (Houck *et al.*, *supra*) into 293 cells and 20-fold concentration (Centricon-10, Amicon) of the 3-day serum-free conditioned medium. In the receptor IgG competition experiments, the concentrated conditioned media were pre-incubated 1 hour at 4° C with receptor IgG.

Without stimulation, 293 cells expressing or not expressing Flt4 showed little or no Flt4 tyrosine phosphorylation. Stimulation of the Flt4-expressing cells by Flt4/IgG antiserum showed the tyrosine phosphorylation of two bands of 180 and 120 kDa. No increase above basal phosphorylation was observed with preimmune serum, and no bands were found with Flt4/IgG antiserum stimulation of non-expressing cells. Two Flt4 bands of about this size have been reported as being expressed by DAMI and HEL cells. Pajusola *et al.*,

Oncogene, 8, *supra*. In addition, SDS gel analysis of purified Flt4/IgG shows that it is composed of peptides of 150, 80, and 70 kDa. N-terminal amino acid sequence of the Flt4/IgG peptides shows that the 150 and 70 kDa bands have the amino acid sequence YSMTPTL (SEQ ID NO: 9) (matching the Flt4 sequence starting at residue 25) and that the 80 kDa band has the sequence SLRRRQQQD (SEQ ID NO: 10) (matching the Flt4 sequence beginning at residue 473). Thus, both the Flt4/IgG and full-length Flt4 appear to be partially cleaved in the extracellular domain, and the tyrosine phosphorylated bands of 180 and 120 kDa observed in the Flt4 phosphorylation assays would correspond to the 150 and 80 kDa peptides of Flt4/IgG. Addition of a polyclonal antiserum to the Flt4 expressing cells showed the tyrosine phosphorylation of two Flt4 bands of 180 and 120 kDa; no bands were observed in non-expressing cells. These data show that polyclonal antibodies generated to the extracellular domain of the Flt4 receptor are capable of activating Flt4 tyrosine phosphorylation.

To determine whether VRP could activate the tyrosine phosphorylation of Flt4, conditioned media from mammalian cells transfected with the VRP expression plasmid was assayed. This conditioned medium stimulated the tyrosine phosphorylation of the same 180 and 120 kDa bands found with the agonist polyclonal antibodies, demonstrating that VRP is able to stimulate the phosphorylation of, as well as bind to, Flt4. Conditioned medium from VEGF-expressing cells failed to activate Flt4 tyrosine phosphorylation.

To confirm the specificity of VRP binding to the receptors of the VEGF family, Flt4/IgG, Flt1/IgG, Flk1/IgG, and Htk/IgG were tested for their ability to compete for VRP-stimulated Flt4 phosphorylation. As expected if VRP is a ligand for Flt4, Flt4/IgG prevented the VRP-stimulated phosphorylation, while Flt1/IgG, Flk1/IgG, and Htk/IgG, a fusion protein from an unrelated tyrosine kinase receptor, had little or no effect. These data show that VRP is able to induce the tyrosine phosphorylation of Flt4.

EXAMPLE 6

Purification of VRP and Binding to Labeled Flt4/IgG

The reading frame encoding the N-terminal secretion signal sequence and about 30 amino acids of the herpes glycoprotein D (Lasky and Dowbenko, DNA, 3: 23-29 [1984]; Pennica *et al.*, Proc. Natl. Acad. Sci. USA, 92: 1142-1146 [1995]) were fused with a short linker sequence to the putative mature sequence of VRP. Following secretion from mammalian cells, this construct is expected to give the N-terminal glycoprotein D sequence:

KYALADASLKMADPNRFRGKDLPLDQLLEGGAHYALLP (SEQ ID NO: 11) followed by the mature VRP sequence GPREAPAAAAAFE (SEQ ID NO: 12). DNA encoding this fusion protein was cloned into the vector pRK5 to give the plasmid pRK.vh1.4.5. This plasmid was transfected into 293 cells by electroporation (Janssen *et al.*, *supra*), and VRP purified from the 3-4 day serum-free conditioned medium by monoclonal antibody (5B6) affinity chromatography and quantitated by colorimetric assay (Bio-Rad). This antibody is specific for the glycoprotein D sequence fused to the N-terminus of VRP.

Flt4/IgG was iodinated to a specific activity of 1000-1500 Ci/mmol with IodobeadsTM brand iodinated beads (Pierce). Binding was performed with ~20,000 cpm ¹²⁵I-Flt4/IgG and 12 ng VH1.4 gD fusion protein in PBS, 0.5% BSA, 0.02% Tween-20TM surfactant, 1 µg/mL heparin (binding buffer) containing 20 µL of a 50% slurry of glass beads conjugated to ~30 µg anti-gD monoclonal antibody (5B6) in a final volume of 100 µL for 4-6 hours at 22°C. Beads were collected by filtration (Millipore Multiscreen-HV), washed five times with 200 µL binding buffer, and counted. For binding at increasing concentrations of Flt4/IgG (Fig. 5B) the binding

buffer was DMEM (low glucose):F12 (50:50), 20 mM sodium HEPES, pH 7.2, 10% fetal bovine serum, 0.2% gelatin, and 1 µg/mL heparin.

The purified VRP specifically bound to ^{125}I -Flt4/IgG, and the binding was not competed by unlabeled Flt1/IgG or Flk1/IgG (Fig. 5A). Binding competition with increasing concentrations of unlabeled Flt4/IgG (Fig. 5B) gave an EC_{50} for this interaction of ~0.7 nM, suggesting that the binding of VRP to Flt4 is of high affinity as would be expected if VRP is a biologically relevant ligand for Flt4.

RNA Blots

Blots containing poly(A)⁺ human RNA were from Clontech. For the G61 glioma cell line, 5 µg of poly(A)⁺ and poly(A)⁻ RNA were electrophoresed on a 1% agarose/2.2 M formaldehyde gel and transferred to nitrocellulose (Janssen *et al.*, *supra*). Blots were hybridized with ^{32}P -labeled probes ovh1.4 and ovh1.5 and washed in 30 mM NaCl/3 mM trisodium citrate at 55°C.

The G61 glioma cell line used in the cloning of VRP expresses a major VRP RNA band of about 2.4 kb. A minor band of about 2.2 kb may also be present. A 2.4 kb band was expressed in adult human tissues from heart, placenta, ovary, and small intestine; a weaker band was found in lung, skeletal muscle, spleen, prostate, testis, and colon. Expression of a 2.4 kb mRNA was also found in fetal lung and kidney.

EXAMPLE 7

Mitogenic Activity of VRP

To test whether VRP has mitogenic activity like that found for VEGF, the growth of human lung endothelial cells was determined at increasing concentrations of VRP or VEGF (Figure 6). Specifically, human lung microvascular endothelial cells (HMVEC-L, Clonetics, San Diego, CA) were maintained in the recommended growth medium (EGM-MV with 5% fetal calf serum). For the assay of mitogenesis, low passage (<6) cells were seeded at 6500 cells/well in 48-well plates (Costar) and maintained overnight in the recommended growth medium. The medium was removed, and the cells were maintained in the growth medium (2% fetal calf serum) without bovine brain extract and supplemented with VEGF or VRP. After four days, the cells were removed with trypsin and counted with a Coulter counter (Hialeah, FL).

VRP promoted the growth of these endothelial cells (see Fig. 6), and thus shares this mitogenic activity with VEGF. This is in contrast to PlGF, which has been reported to lack such mitogenic activity (at ≤ 35 nM). Park *et al.*, *supra*. While an effective mitogenic agent, VRP was about 100 fold less potent than VEGF in this assay.

In conclusion, a novel secreted protein, VRP, has now been identified that is a Flt4 ligand and that stimulates the tyrosine phosphorylation of the receptor tyrosine kinase Flt4. VRP is a third member of the VEGF protein family and has about 30% amino acid identity with VEGF and PlGF. In addition to the VEGF-like domain, VRP contains a ~180 amino acid C-terminal, cysteine-rich domain not found in other members of the VEGF family. VRP fails to interact appreciably with the VEGF receptors Flt1 and Flk1.

Deposit of Material

The following plasmid has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

	<u>Plasmid</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
5	pRK.vh1.4.1	97249	September 6, 1995

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement
10 between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886
15 OG 638).

The assignee of the present application has agreed that if a culture of the plasmid on deposit should die or be lost or destroyed when cultivated under suitable conditions, the plasmid will be promptly replaced on notification with another of the same plasmid. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in
20 accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not
25 constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: VEGF-Related Protein

5 (iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
10 (D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
15 (B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
20 (B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lee, Wendy M.
(B) REGISTRATION NUMBER: P-40,378
25 (C) REFERENCE/DOCKET NUMBER: P0963PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 415/225-1994
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

30 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2031 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
35 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCGGGGTGT TCTGGTGTCC CCCGCCCCGC CTCTCCAAA AGCTACACCG 50
ACGCGGACCG CGGCGGCGTC CTCCTCGCC CTCGCTTCAC CTCGCGGGCT 100
CCGAATGCGG GGAGCTCGGA TGTCCGGTTT CCTGTGAGGC TTTTACCTGA 150
40 CACCCGCCGC CTTTCCCCGG CACTGGCTGG GAGGGCGCCC TGCAAAGTTG 200

GGAACGCGGA GCCCCGGACC CGCTCCCGCC GCCTCCGGCT CGCCCAGGGG 250
 GGGTCGCCCG GAGGAGCCCG GGGGAGAGGG ACCAGGAGGG GCCCCGCGCC 300
 TCGCAGGGGC GCCCCGCCCC CCACCCCTGC CCCC GCCAGC GGACCGGTCC 350
 CCCACCCCGG GTCCTTCCAC CATGCACTTG CTGGGCTTCT TCTCTGTGGC 400
 5 GTGTTCTCTG CTCGCCGCTG CGCTGCTCCC GGTCTCTCGC GAGGCGCCCG 450
 CCGCGCGCCG CGCCTTCGAG TCCGGACTCG ACCTCTCGGA CGCGGAGCCC 500
 GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG AGGAGCAGTT 550
 ACGGTCTGTG TCCAGTGTAG ATGAACTCAT GACTGTACTC TACCCAGAAT 600
 ATTGGAATAT GTACAAGTGT CAGCTAAGGA AAGGAGGCTG GCAACATAAC 650
 10 AGAGAACAGG CCAACCTCAA CTCAAGGACA GAAGAGACTA TAAATTTTGC 700
 TGCAGCACAT TATAATACAG AGATCTTGAA AAGTATTGAT AATGAGTGGA 750
 GAAAGACTCA ATGCATGCCA CGGGAGGTGT GTATAGATGT GGGGAAGGAG 800
 TTTGGAGTCG CGACAAACAC CTTCTTTAAA CCTCCATGTG TGTCCGTCTA 850
 CAGATGTGGG GGTGCTGCA ATAGTGAGGG GCTGCAGTGC ATGAACACCA 900
 15 GCACGAGCTA CCTCAGCAAG ACGTTATTTG AAATTACAGT GCCTCTCTCT 950
 CAAGGCCCCA AACCAGTAAC AATCAGTTTT GCCAATCACA CTTCTGCCC 1000
 ATGCATGTCT AACTGGATG TTTACAGACA AGTTCATTCC ATTATTAGAC 1050
 GTTCCCTGCC AGCAACACTA CCACAGTGTG AGGCAGCGAA CAAGACCTGC 1100
 CCCACCAATT ACATGTGGAA TAATCACATC TGCAGATGCC TGGCTCAGGA 1150
 20 AGATTTTATG TTTTCCTCGG ATGCTGGAGA TGAATCAACA GATGGATTCC 1200
 ATGACATCTG TGGACCAAAC AAGGAGCTGG ATGAAGAGAC CTGTCAGTGT 1250
 GTCTGCAGAG CGGGGCTTCG GCCTGCCAGC TGTGGACCCC ACAAGAAGT 1300
 AGACAGAAAC TCATGCCAGT GTGTCTGTAA AAACAAACTC TTCCCAGCC 1350
 AATGTGGGGC CAACCGAGAA TTTGATGAAA ACACATGCCA GTGTGTATGT 1400
 25 AAAAGAACCT GCCCCAGAAA TCAACCCCTA AATCCTGGAA AATGTGCCTG 1450
 TGAATGTACA GAAAGTCCAC AGAAATGCTT GTTAAAGGA AAGAAGTTCC 1500
 ACCACCAAAC ATGCAGCTGT TACAGACGGC CATGTACGAA CCGCCAGAAG 1550
 GCTTGTGAGC CAGGATTTTC ATATAGTGAA GAAGTGTGTC GTTGTGTCCC 1600
 TTCATATTGG AAAAGACCAC AAATGAGCTA AGATTGTACT GTTTTCCAGT 1650

TCATCGATTT TCTATTATGG AAAACTGTGT TGCCACAGTA GAACTGTCTG 1700
TGAACAGAGA GACCCTTGTG GGTCCATGCT AACAAAGACA AAAGTCTGTC 1750
TTTCCTGAAC CATGTGGATA ACTTTACAGA AATGGACTGG AGCTCATCTG 1800
CAAAAGGCCT CTTGTAAAGA CTGGTTTTCT GCCAATGACC AAACAGCCAA 1850
5 GATTTTCCTC TTGTGATTTC TTTAAAAGAA TGA CTATATA ATTTATTTCC 1900
ACTAAAATA TTGTTTCTGC ATTCATTTTT ATAGCAACAA CAATTGGTAA 1950
AACTCACTGT GATCAATATT TTTATATCAT GCAAATATG TTAAAATAA 2000
AATGAAAATT GTATTAAAAA AAAAAAAAAA A 2031

(2) INFORMATION FOR SEQ ID NO:2:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2031 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

- 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTTTTTTT TTTTAAATA CAATTTTCAT TTTATTTTAA ACATATTTTG 50
CATGATATAA AAATATTGAT CACAGTGAGT TTTACCAATT GTTGTGCTA 100
TAAAAATGAA TGCAGAAACA ATATTTT TAG TGGAAATAA TTATATAGTC 150
ATTCTTTTAA AGAAATCACA AGAGGAAAAT CTTGGCTGTT TGGTCATTGG 200
20 CAGAAAACCA GTCTTTACAA GAGGCCTTTT GCAGATGAGC TCCAGTCCAT 250
TTCTGTAAAG TTATCCACAT GGTTCAGGAA AGACAGACTT TTGTCTTGT 300
TAGCATGGAC CCACAAGGGT CTCTCTGTTT ACAGACAGTT CTACTGTGGC 350
AACACAGTTT TCCATAATAG AAAATCGATG AACTGGAAAA CAGTACAATC 400
TTAGCTCATT TGTGGTCTTT TCCAATATGA AGGGACACAA CGACACACTT 450
25 CTTCACTATA TGAAAATCCT GGCTCACAAG CCTTCTGGCG GTTCGTACAT 500
GGCCGTCTGT AACAGCTGCA TGTTTGGTGG TGGAACCTCT TTCCTTTTAA 550
CAAGCATTTT TGTGGACTTT CTGTACATTC ACAGGCACAT TTTCCAGGAT 600
TTAGGGGTTG ATTTCTGGGG CAGGTTCTTT TACATACACA CTGGCATGTG 650
TTTTCATCAA ATTCTCGGTT GGCCCCACAT TGGCTGGGGA AGAGTTTGT 700
30 TTACAGACA CACTGGCATG AGTTTCTGTC TAGTTCTTTG TGGGGTCCAC 750
AGCTGGCAGG CCGAAGCCCC GCTCTGCAGA CACACTGACA GGTCTCTTCA 800

TCCAGCTCCT TGT TTGGTCC ACAGATGTCA TGAATCCAT CTGTTGAGTC 850
 ATCTCCAGCA TCCGAGGAAA ACATAAAATC TTCTGAGCC AGGCATCTGC 900
 AGATGTGATT ATTCCACATG TAATTGGTGG GGCAGGTCTT GTTCGCTGCC 950
 TGACACTGTG GTAGTGTTC TGGCAGGGAA CGTCTAATAA TGAATGAAC 1000
 5 TTGTCTGTAA ACATCCAGTT TAGACATGCA TCGGCAGGAA GTGTGATTGG 1050
 CAAACTGAT GTTACTGGT TTGGGGCCTT GAGAGAGAGG CACTGTAATT 1100
 TCAAATAACG TCTTGCTGAG GTAGCTCGTG CTGGTGTTC TCACTGCAG 1150
 CCCCTCACTA TTGCAGCAAC CCCCATCTCT GTAGACGGAC ACACATGGAG 1200
 GTTTAAAGAA GGTGTTTGTC GCGACTCCAA ACTCCTTCCC CACATCTATA 1250
 10 CACACCTCCC GTGGCATGCA TTGAGTCTTT CTCCACTCAT TATCAATACT 1300
 TTTCAAGATC TCTGTATTAT AATGTGCTGC AGCAAATTTT ATAGTCTCTT 1350
 CTGTCCTTGA GTTGAGGTTG GCCTGTTCTC TGTATGTTG CCAGCCTCCT 1400
 TTCCTTAGCT GACACTTGTA CATTTTCCAA TATCTGGGT AGAGTACAGT 1450
 CATGAGTTCA TCTACACTGG ACACAGACCG TAACTGCTCC TCCAGATCTT 1500
 15 TGCTTGATA AGCCGTGGCC TCGCCCGCGT CGGGCTCCGC GTCCGAGAGG 1550
 TCGAGTCCGG ACTCGAAGGC GCGGGCGGCG GCGGGCGCCT CGCGAGGACC 1600
 CGGGAGCAGC ACAGCGGCGA GCAGAGAACA CGCCACAGAG AAGAAGCCCA 1650
 GCAAGTGCAT GGTGGAAGGA CCGGGGTGG GGGACCGTC CGCTGGCGGG 1700
 GGCAGGGGTG GGGGCGCGG CGCCCTGCG AGGCCGCGG CCCCTCCTGG 1750
 20 TCCCTCTCCC CCGGGCTCCT CCCGGCGACC CCCCTGGGC GAGCCGGAGG 1800
 CGGCGGGAGC GGTCCGGGG CTCCGCTTC CCAACTTGC AGGGCGCCCT 1850
 CCCAGCCAGT ACCGGGGAAA GCGGCGGGT GTCAGGTAAA AGCCTCACAG 1900
 GAAACCGGAC ATCCGAGCTC CCCGATTTC GAGCCCGCGA GGTGAAGCGA 1950
 GGGCGAGGGA GGACCGCGC GCGGTCCGCG TCGGTGTAGC TTTTGGAGA 2000
 25 GCGGGGCGG GGGACACCAG AACACCCCGC G 2031

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 419 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala	
	1 5 10 15	
	Ala Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala	
	20 25 30	
5	Ala Phe Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala	
	35 40 45	
	Gly Glu Ala Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu	
	50 55 60	
10	Arg Ser Val Ser Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro	
	65 70 75	
	Glu Tyr Trp Lys Met Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp	
	80 85 90	
	Gln His Asn Arg Glu Gln Ala Asn Leu Asn Ser Arg Thr Glu Glu	
	95 100 105	
15	Thr Ile Lys Phe Ala Ala Ala His Thr Asn Thr Glu Ile Leu Lys	
	110 115 120	
	Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro Arg Glu	
	125 130 135	
20	Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val Ala Thr Asn Thr	
	140 145 150	
	Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys	
	155 160 165	
	Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Ser Tyr	
	170 175 180	
25	Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly	
	185 190 195	
	Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg	
	200 205 210	
	Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile	
30	215 220 225	
	Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn	
	230 235 240	
	Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg	
	245 250 255	
35	Cys Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp	
	260 265 270	
	Asp Ser Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu	
	275 280 285	
	Leu Asp Glu Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg	

	290	295	300
	Pro Ala Ser Cys Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys		
	305	310	315
5	Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala		
	320	325	330
	Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys Lys Arg		
	335	340	345
	Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys		
	350	355	360
10	Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly Lys Lys		
	365	370	375
	Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr Asn		
	380	385	390
15	Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val		
	395	400	405
	Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser		
	410	415	419

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 147 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25	Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu		
	1	5	10 15
	Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala		
	20	25	30
	Glu Gly Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp		
	35	40	45
30	Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp		
	50	55	60
	Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro		
	65	70	75
35	Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu		
	80	85	90
	Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln		
	95	100	105
	Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met		
	110	115	120

Ser Phe Leu Gln His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp
125 130 135

Arg Ala Arg Gln Glu Lys Cys Asp Lys Pro Arg Arg
140 145 147

5 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Pro	Val	Met	Arg	Leu	Phe	Pro	Cys	Phe	Leu	Gln	Leu	Leu	Ala	
1				5					10					15	
Gly	Leu	Ala	Leu	Pro	Ala	Val	Pro	Pro	Gln	Gln	Trp	Ala	Leu	Ser	
				20					25					30	
Ala	Gly	Asn	Gly	Ser	Ser	Glu	Val	Glu	Val	Val	Pro	Phe	Gln	Glu	
				35					40					45	
Val	Trp	Gly	Arg	Ser	Tyr	Cys	Arg	Ala	Leu	Glu	Arg	Leu	Val	Asp	
				50					55					60	
Val	Val	Ser	Glu	Tyr	Pro	Ser	Glu	Val	Glu	His	Met	Phe	Ser	Pro	
20				65					70					75	
Ser	Cys	Val	Ser	Leu	Leu	Arg	Cys	Thr	Gly	Cys	Cys	Gly	Asp	Glu	
				80					85					90	
Asn	Leu	His	Cys	Val	Pro	Val	Glu	Thr	Ala	Asn	Val	Thr	Met	Gln	
				95					100					105	
Leu	Leu	Lys	Ile	Arg	Ser	Gly	Asp	Arg	Pro	Ser	Tyr	Val	Glu	Leu	
				110					115					120	
Thr	Phe	Ser	Gln	His	Val	Arg	Cys	Glu	Cys	Arg	Pro	Leu	Arg	Glu	
				125					130					135	
Lys	Met	Lys	Pro	Glu	Arg	Cys	Gly	Asp	Ala	Val	Pro	Arg	Arg		
30				140					145					149	

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 299 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGCTCTACAG ATGTGGGGGT TGCTGCAATA GTGAGGGGCT GCAGTGCATG 50

AACACCAGCA CGAGCTACCT CAGNAAGACG TTATTTGAAA TTACAGTGCC 100
 TCTCTCTCAA GGGCCCAAAC CAGTAACAAT CAGTTTTGCC AATCACACTT 150
 CCTGCCGATG CATGTCTAAA CTGGATGTTT ACAGACAAGT TCATTCCATT 200
 ATTAGACGTT CCCTGCCAGC AACACTACCA CAGTGTGAGG CAGCGAACAA 250
 5 GACCTGCCCC ACCAATTACA TGTGGAATAA TCACATCTGC AGATGCCTG 299

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 base pairs
 (B) TYPE: Nucleic Acid
 10 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGGTGTTCA TGCACTGCAG CCCCTCACTA TTGCAGCAAC CCCACATCT 50

(2) INFORMATION FOR SEQ ID NO:8:

- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCACTGCGAG ATGTGATTAT TCCACATGTA ATTGGTGGGG CAGGTCTTGT 50

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 25 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Ser Met Thr Pro Pro Thr Leu
 1 5 8

30 (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser Leu Arg Arg Arg Gln Gln Gln Asp

1 5 9

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 40 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Tyr Ala Leu Ala Asp Ala Ser Leu Lys Met Ala Asp Pro Asn
1 5 10 15
10 Arg Phe Arg Gly Lys Asp Leu Pro Val Leu Asp Gln Leu Leu Glu
20 25 30
Gly Gly Ala Ala His Tyr Ala Leu Leu Pro
35 40

(2) INFORMATION FOR SEQ ID NO:12:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

20 Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe Glu
1 5 10 13

WHAT IS CLAIMED IS:

1. Isolated biologically active human VEGF-related protein (VRP) containing at least 265 amino acids.
2. The protein of claim 1 containing 265 to about 450 amino acids.
- 5 3. The protein of claim 1 containing about 300-450 amino acids.
4. The protein of claim 1 containing about 350-450 amino acids.
5. The protein of claim 1 containing about 399-419 amino acids.
6. The protein of claim 1 comprising an amino acid sequence having at least residues +1 through 29, inclusive, of Figure 1.
- 10 7. The protein of claim 6 comprising an amino acid sequence having at least residues +1 through 137, inclusive, of Figure 1.
8. The protein of claim 6 comprising an amino acid sequence having at least residues -20 through 29, inclusive, of Figure 1.
9. The protein of claim 6 comprising an amino acid sequence having at least residues -20 through 15 137, inclusive, of Figure 1.
10. Isolated biologically active human VEGF-related protein (VRP) comprising an amino acid sequence comprising at least residues +1 through 29, inclusive, of Figure 1.
11. The protein of claim 10 comprising an amino acid sequence having at least residues +1 through 137, inclusive, of Figure 1.
- 20 12. The protein of claim 10 comprising an amino acid sequence having at least residues -20 through 29, inclusive, of Figure 1.
13. The protein of claim 10 comprising an amino acid sequence having at least residues -20 through 137, inclusive, of Figure 1.
14. Isolated biologically active human VEGF-related protein (VRP) comprising an amino acid 25 sequence shown as residues -20 through 399, inclusive, or residues 1 through 399, inclusive, of Figure 1.
15. The protein of claim 14 wherein the sequence is shown as -20 through 399, inclusive, of Figure 1.
16. The protein of claim 14 wherein the sequence is shown as 1 through 399, inclusive, of Figure 1.
- 30 17. A composition comprising the protein of claim 1 and a pharmaceutically acceptable carrier.
18. A pharmaceutical composition useful for promotion of vascular endothelial cell growth comprising a therapeutically effective amount of the protein of claim 1 in a pharmaceutically acceptable carrier.
19. The composition of claim 18 further comprising a cell growth factor other than said protein.
20. A method for treating trauma affecting the vascular endothelium comprising administering to 35 a mammal suffering from said trauma an effective amount of the composition of claim 18.
21. The method of claim 20 further comprising administering to said mammal an effective amount of a cell growth factor other than said protein.

22. A method for treating a dysfunctional state characterized by lack of activation or lack of inhibition of a receptor for VRP in a mammal comprising administering to the mammal an effective amount of the composition of claim 17.
23. A method for stimulating the phosphorylation of a tyrosine kinase domain of a Flt4 receptor comprising contacting an extracellular domain of the Flt4 receptor with the protein of claim 1.
24. A chimeric polypeptide comprising the protein of claim 1 fused to a tag polypeptide sequence.
25. A monoclonal antibody which binds to the protein of claim 1 and neutralizes a biological activity of the protein.
26. The antibody of claim 25 wherein the biological activity of the protein is promoting neovascularization or vascular permeability or vascular endothelial cell growth in a mammal.
27. A composition comprising the antibody of claim 25 and a pharmaceutically acceptable carrier.
28. A method of treating diseases or disorders characterized by undesirable excessive neovascularization or vascular permeability in a mammal comprising administering to said mammal an effective amount of the composition of claim 27.
29. A method for treating a dysfunctional state characterized by excessive activation or inhibition of a receptor for VRP in a mammal comprising administering to the mammal an effective amount of the composition of claim 27.
30. A monoclonal antibody which binds to the N-terminal portion from residues -20 through 137, inclusive, or from residues 1 through 137, inclusive, of the amino acid sequence shown in Figure 1.
31. A peptide consisting of an amino acid sequence shown as residues -20 through -1, inclusive, of Figure 1.
32. A method for treating a dysfunctional state characterized by excessive activation or inhibition of a receptor for VRP in a mammal comprising administering to the mammal an effective amount of a VRP antagonist.
33. A method for treating Kaposi's sarcoma in a mammal comprising administering to the mammal an effective amount of a VRP antagonist.
34. An isolated nucleic acid molecule encoding the protein of any one of claims 1-16
35. The nucleic acid molecule of claim 34 further comprising a promoter operably linked to the nucleic acid molecule.
36. A vector comprising the nucleic acid molecule of claim 34.
37. An expression vector comprising the nucleic acid molecule of claim 34 operably linked to control sequences recognized by a host cell transformed with the vector.
38. A host cell comprising the nucleic acid molecule of claim 34.
39. A method of producing VRP comprising culturing the host cell of claim 38 and recovering VRP from the host cell culture.

FIGURE 1B

601 ATTGGAAAAT GTACAAGTGT CAGCTAAGGA AAGGAGGCTG GCAACATAAC AGAGAACAGG
 TAACCTTTTA CATGTTTACA GTCGATTCTT TTCCTCCGAC CGTTGTATTG TCTCTTGTC
 58 W K M Y K C Q L R K G G W Q H N R E Q A

661 CCAACCTCAA CTCAAGGACA GAAGAGACTA TAAAATTTGC TGCAGCACAT TATAATACAG
 GGTGGAGTT GAGTTCCTGT CTTCTCTGAT ATTTTAAACG ACGTCGTGTA ATATTATGTC
 78 N L N S R T E E T I K F A A A H T N T E

sphI

721 AGATCTTGAA AAGTATTGAT AATGAGTGGA GAAAGACTCA ATGCATGCCA CGGGAGGTGT
 TCTAGAACTT TTCATACTA TTACTCACCT CTTTCTGAGT TACGTACGGT GCCCTCCACA
 98 I L K S I D N E W R K T Q C M P R E V C

781 GTATAGATGT GGGGAAGGAG TTTGGAGTCG CGACAAACAC CTTCTTTAAA CCTCCATGTG
 CATATCTACA CCCCTTCCTC AAACCTCAGC GCTGTTTGTG GAAGAAATTT GGAGGTACAC
 118 I D V G K E F G V A T N T F F K P P C V

accI

841 TGTCCGTCTA CAGATGTGGG GGTGCTGCA ATAGTGAGGG GCTGCAGTGC ATGAACACCA
 ACAGGCAGAT GTCTACACCC CCAACGACGT TATCACTCCC CGACGTCACG TACTTGTGGT
 138 S V Y R C G G C C N S E G L Q C M N T S

901 GCACGAGCTA CCTCAGCAAG ACGTTATTG AAATTACAGT GCCTCTCTCT CAAGGCCCCA
 CGTGCTCGAT GGAGTCGTTT TGCAATAAAC TTAAATGTCA CGGAGAGAGA GTTCCGGGGT
 158 T S Y L S K T L F E I T V P L S Q G P K

961 AACCAGTAAC AATCAGTTTT GCCAATCACA CTTCTGCGG ATGCATGTCT AAACCTGGATG
 TTGGTCATTG TTAGTCAAAA CGGTTAGTGT GAAGGACGGC TACGTACAGA TTTGACCTAC
 178 P V T I S F A N H T S C R C M S K L D V

1021 TTTACAGACA AGTTCATTCC ATTATTAGAC GTTCCCTGCC AGCAACACTA CCACAGTGTG
 AAATGTCTGT TCAAGTAAGG TAATAATCTG CAAGGGACGG TCATTGTGAT GGTGTCACAG
 198 Y R Q V H S I I R R S L P A T L P Q C \ Q

1081 AGGCAGCGAA CAAGACCTGC CCCACCAATT ACATGTGGAA TAATCACATC TGCAGATGCC
 TCCGTCGCTT GTTCTGGACG GGGTGGTTAA TGTACACCTT ATTAGTGTAG ACGTCTACGG
 218 A A N K T C P T N Y M W N N H I C R C L

FIGURE 1C

1141 TGGCTCAGGA AGATTTTATG TTTTCCTCGG ATGCTGGAGA TGA CTCAACA GATGGATTCC
 ACCGAGTCCT TCTAAAATAC AAAAGGAGCC TACGACCTCT ACTGAGTTGT CTACCTAAGG
 238 A Q E D F M F S S D A G D D S T D G F H

1201 ATGACATCTG TGGACCAAAC AAGGAGCTGG ATGAAGAGAC CTGTCAGTGT GTCTGCAGAG
 TACTGTAGAC ACCTGGTTTG TTCCTCGACC TACTTCTCTG GACAGTCACA CAGACGTCTC
 258 D I C G P N K E L D E E T C Q C V C R A

1261 CGGGGCTTCG GCCTGCCAGC TGTGGACCCC ACAAGA AACT AGACAGAAAC TCATGCCAGT
 GCCCCGAAGC CGGACGGTCG ACACCTGGGG TGTTCCTTGA TCTGTCTTTG AGTACGGTCA
 278 G L R P A S C G P H K E L D R N S C Q C

1321 GTGTCTGTAA AAACAACTC TTCCCCAGCC AATGTGGGGC CAACCGAGAA TTTGATGAAA
 CACAGACATT TTTGTTTGAG AAGGGGTCGG TTACACCCCG GTTGGCTCTT AAAC TACTTT
 298 V C K N K L F P S Q C G A N R E F D E N

1381 ACACATGCCA GTGTGTATGT AAAAGAACCT GCCCCAGAAA TCAACCCCTA AATCCTGGAA
 TGTGTACGGT CACACATACA TTTTCTTGGA CGGGGTCTTT AGTTGGGGAT TTAGGACCTT
 318 T C Q C V C K R T C P R N Q P L N P G K

1441 AATGTGCCTG TGAATGTACA GAAAGTCCAC AGAAATGCTT GTTAAAAGGA AAGAAGTTCC
 TTACACGGAC ACTTACATGT CTTTCAGGTG TCTTTACGAA CAATTTTCCT TTCTTCAAGG
 338 C A C E C T E S P Q K C L L K G K K F H

eaeI

1501 ACCACCAAAC ATGCAGCTGT TACAGACGGC CATGTACGAA CCGCCAGAAG GCTTGTGAGC
 TGGTGGTTTG TACGTCGACA ATGTCTGCCG GTACATGCTT GGCGGTCTTC CGAACACTCG
 358 H Q T C S C Y R R P C T N R Q K A C E P

1561 CAGGATTTTC ATATAGTGAA GAAGTGTGTC GTTGTGTCCC TTCATATTGG AAAAGACCAC
 GTCCTAAAAG TATATCACTT CTTACACAG CAACACAGGG AAGTATAACC TTTTCTGGTG
 378 G F S Y S E E V C R C V P S Y W K R P Q

claI

1621 AAATGAGCTA AGATTGTACT GTTTTCAGT TCATCGATT TCTATTATGG AAAACTGTGT
 TTTACTCGAT TCTAACATGA CAAAAGGTCA AGTAGCTAAA AGATAATACC TTTTGACACA
 398 M S O

1681 TGCCACAGTA GAACTGTCTG TGAACAGAGA GACCCTGTG GGTCCATGCT AACAAAGACA
 ACGGTGTCAT CTTGACAGAC ACTTGTCTCT CTGGGAACAC CCAGGTACGA TTGTTTCTGT

FIGURE 1D

sstI

1741 AAAGTCTGTC TTTCCTGAAC CATGTGGATA ACTTTACAGA AATGGACTGG AGCTCATCTG
TTTCAGACAG AAAGGACTTG GTACACCTAT TGAAATGTCT TTACCTGACC TCGAGTAGAC

1801 CAAAAGGCCT CTTGTAAAGA CTGGTTTTCT GCCAATGACC AAACAGCCAA GATTTTCCTC
GTTTCCGGA GAACATTTCT GACCAAAGA CGGTACTGG TTTGTCGGTT CTAAAAGGAG

1861 TTGTGATTC TTAAAAGAA TGACTATATA ATTTATTTCC ACTAAAAATA TTGTTTCTGC
AACACTAAAG AAATTTTCTT ACTGATATAT TAAATAAAGG TGATTTTAT AACAAAGACG

1921 ATTCATTTT ATAGCAACAA CAATTGGTAA AACTCACTGT GATCAATATT TTTATATCAT
TAAGTAAAAA TATCGTTGTT GTTAACCATT TTGAGTGACA CTAGTTATAA AAATATAGTA

1981 GCAAATATG TTAAAATAA AATGAAATT GTATTAAAA AAAAAAAAAA A
CGTTTATAC AAATTTTATT TTACTTTTAA CATAATTTT TTTTTTTTTT T

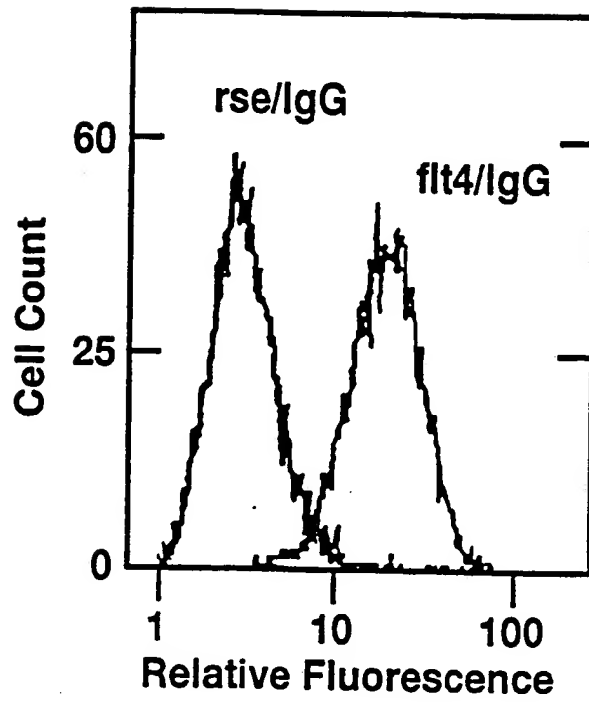
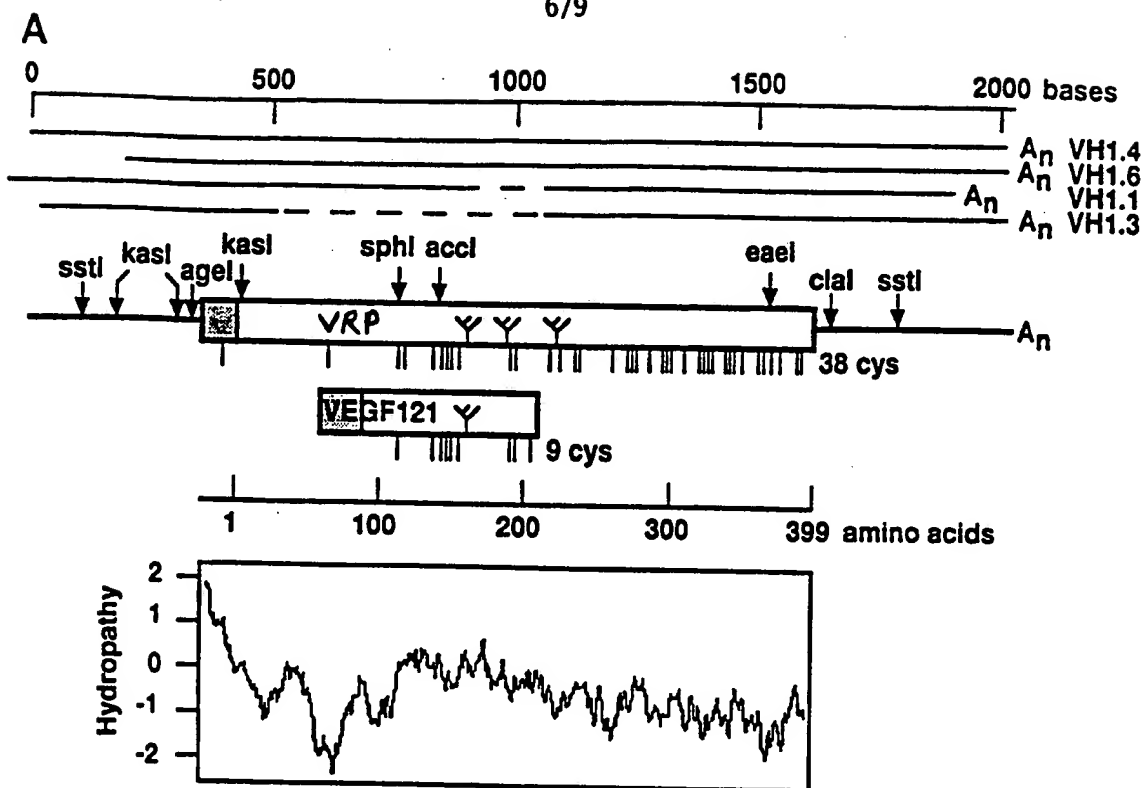


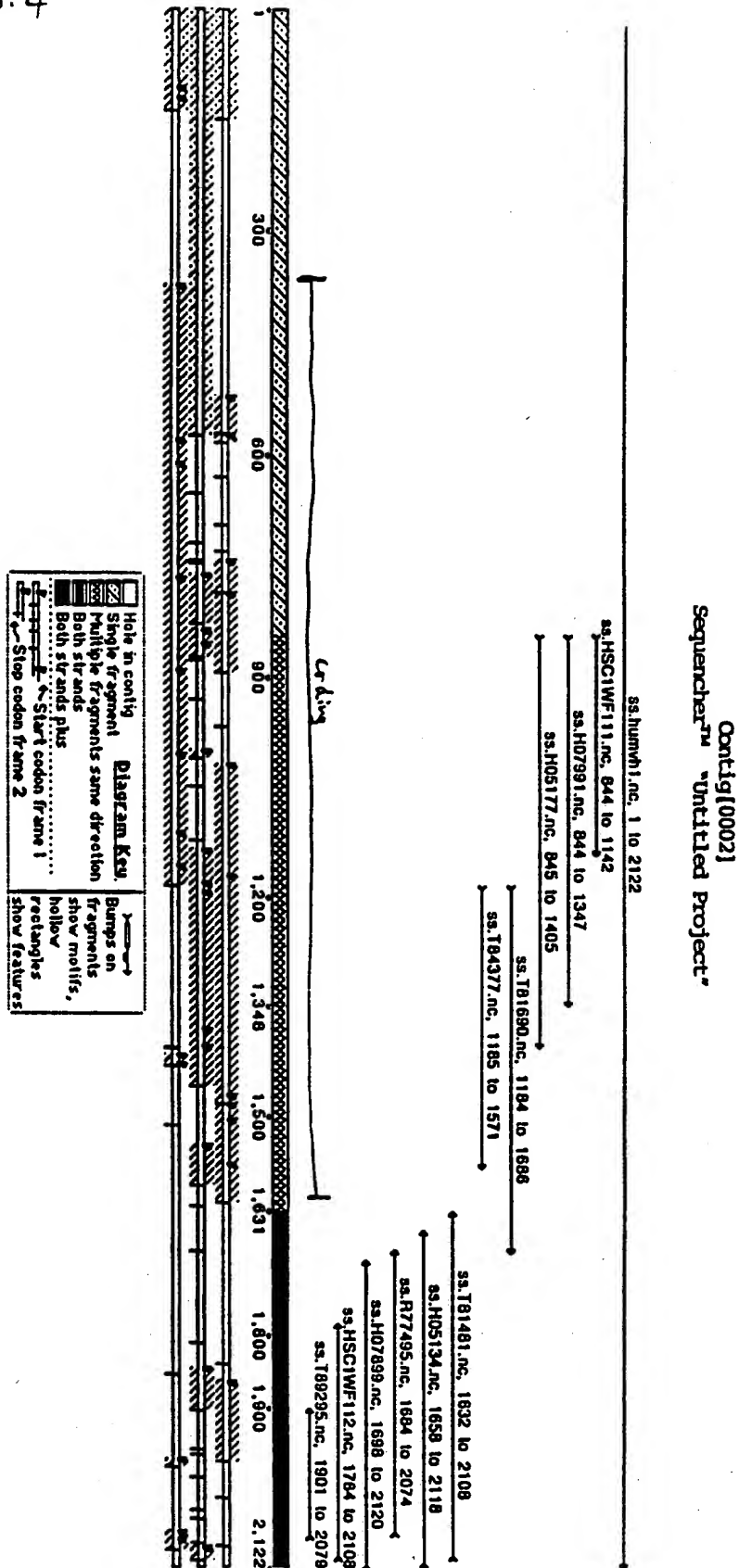
Figure 2

**B**

VRP	-20	MHLLGFFSVACSL	LAAALLPGPREAPAAAAAFESGLDLS	DAEPDAGEATA	
VRP	31	YASKDLEEQLRSVSSVDELM	TVLYPEYWK	MYKCOLRKGGWQHNR	EQANLN
VEGF121	-26	MNFLLSWVHWSL	ALLYLHHA
PIGF131	-18	MPVMRLFPCLQL	LAGLALPA
VRP	81	SRTETIKF	AAAHYNTEILKSIDNEW	RKTQCMPREVCIDVGKE	FGVATNT
VEGF121	-5	KWS	AAPMAEGGGQNHHEVVK	FMDVYORSYCHPIET	LVDIFOEYPOEIEY
PIGF131	4	VPP	QOWALSAGNGSSEVEVVP	FOEVWGRSYCRALER	LVDVSEYPSVEH
VRP	131	FFKPPCVSVY	RCGGCCNSEGLQCMNTSTSYLSKT	LFEITVPLS	OGPKPMT
VEGF121	46	IFKPS	CVPLMRCGGCCNDEGLE	CVPTESNITMQIMRI	KP..HGGOHIGE
PIGF131	54	MFSP	SCVSLLRCTGCCGDENL	HCVPVETANVTMOLLKIRS	..GDRPSYVE
VRP	181	ISFANHTS	CRCMSKLDVYRQVHS	IIRRLSLPATLP	QCQAANKTCPTNYMW
VEGF121	94	MSFL	QHNKCECRPKKDRAR	OEKCDKPRR	
PIGF131	102	LTF	SDHVRCECRPLREKMKPER	CGDAVPRR	
VRP	231	NHICRCLAQED	FMFSSDAGDDSTDGFHD	ICGPNKELDEETCQCVC	RAGLR
VRP	281	PASCGPHKELDRNS	CQCVCCKNKLFP	SQCGANREFDENTCOCVCK	RTCPRN
VRP	331	OPLNPGKCACECTES	POKCLLKGKKFHHOTC	SCYRRPCTNRQKACE	PGFS
VRP	381	YSEEV	CRCPVPSYWKRP	OMS	

Figure 3

FIG. 4



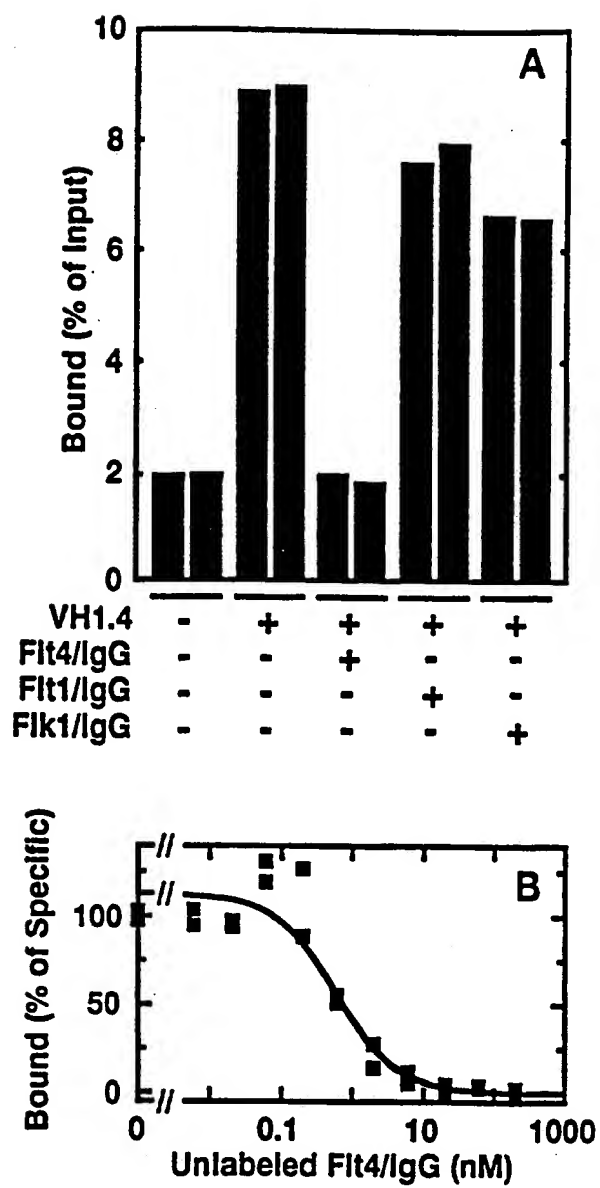
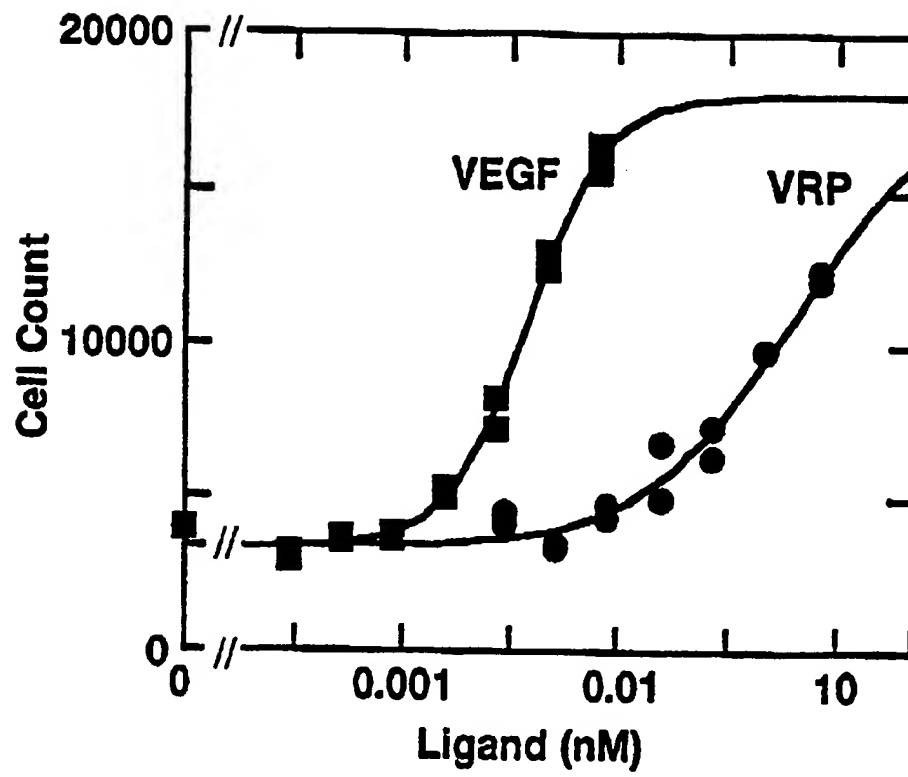


Figure 5

FIG. 6



A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/19 C07K14/52 C07K19/00 C07K16/24 A61K38/19 G01N33/50		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ONCOGENE, vol. 10, 1995, pages 973-984, XP002022269 J.-P. BORG ET AL.: "Biochemical characterization of two isoforms of FLT4, a VEGF receptor-related tyrosine kinase" cited in the application see the whole document, especially the abstract. --- -/--	1
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 23 January 1997		Date of mailing of the international search report 12.02.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patendaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer Yeats, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ONCOGENE, vol. 9, 1994, pages 3545-3555, XP002022270 K. PAJUSOLA ET AL.: "Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors" cited in the application see the whole document, in particular the abstract, introduction and discussion. ---	1
P,X	WO,A,95 24473 (HUMAN GENOME SCIENCES, INC.) 14 September 1995 see the whole document, especially Figure 1 and the claims. ---	1-39
P,X	EMBO J., vol. 15, 1996, pages 290-298, XP002022272 V. JOUKOV ET AL.: "A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGF-2) receptor tyrosine kinase" see the whole document. ---	1-39
P,X	PROC. NATL. ACAD. SCI. USA, vol. 93, 1996, pages 1988-1992, XP002022357 J. LEE ET AL.: "Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4" see the whole document. -----	1-39

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9524473	14-09-95	AU-A- 7394194	25-09-95
		EP-A- 0751992	08-01-97
		ZA-A- 9403464	20-11-95

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.